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Symbiont community assembly shaped by insecticide exposure and feedback on insecticide resistance of *Spodoptera frugiperda*

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Exploring the mechanism of microbiota assembly and its ecological consequences is crucial for connecting microbiome variation to ecosystem function. However, the influencing factors underlying microbiota assembly in the host-microbe system and their impact on the host phenotype remain unclear. Through investigating the prevalent and worsening ecological phenomenon of insecticide resistance in global agriculture, we found that insecticide exposure significantly changed the gut microbiota assembly patterns of a major agricultural invasive insect pest, *Spodoptera frugiperda*. The relative importance of various microbiota assembly processes significantly varied with habitat heterogeneity and heterogeneous selection serving as a potential predictor of the host's insecticide resistance in field populations. Moreover, disturbance of the gut microbiota assembly through antibiotics was revealed to significantly affect the rate and heritability of insecticide resistance evolution, leading to a delay in insecticide resistance evolution in this insect pest. These findings indicate that the gut microbiota assembly process of the insect host is influenced by persistent exposure to habitat conditions, particularly insecticides. This variation in insecticide exposure-related community assembly process subsequently influences the insect host's insecticide resistance phenotype. This study provides insights into gut microbiota assembly processes from a symbiotic perspective and underscores the significant impact of symbiotic community changes on host phenotypic variation.

Environmental and animal microbiota (i.e., microbial communities) are influenced by four fundamentals ecological processes: diversification, selection, drift, and dispersal[1, 2]. Deterministic processes in ecology often involve non-random factors such as environmental filtering and biotic interactions like competition, facilitation, mutualism, and predation[3]. Stochastic processes encompass probabilistic diffusion, random species formation and extinction, and ecological drift[4]. Recently, researchers have begun to deconstruct and assess the significance of these ecological processes using null model analysis, which establishes a statistical framework to investigate and quantify the effects of different ecological processes on microbial community structure, succession, and biogeography. Through the use of with null-model-based phylogenetic and taxonomic β-diversity metrics such as β nearest taxon index (βNTI), beta net relatedness index (βNRI), and Raup-Crick Bray-Curtis distance (RC_{Bray}), the mechanisms driving changes in phylogenetic and taxonomic diversity can be identified using β diversity indices[5]. Ning et al., present a novel pipeline to quantitatively infer community assembly mechanisms through phylogenetic bin-based null model analysis (iCAMP), which can quantify relative importance of five assembly processes including, homogeneous selection (HoS), heterogeneous selection (HeS), homogenizing dispersal (HD), dispersal limitation (DL) and drift (DR)[6]. By employing these approaches, the assembly process of ecological models can be measured, enabling a more precise differentiation between the roles of stochastic and deterministic processes in

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microbial communities, thus enhancing our comprehension of the mechanisms governing microbiota assembly.

Usually, microbiota assembly related ecological processes can be influenced by biotic or abiotic factors[7]. However, while these analyses are frequently applied in modeling environmental microbiomes, their use in investigating the assembly mechanisms of symbiont communities in animals is limited[6]. Several recent studies have shown that microbiota communities of insects can be partially driven by deterministic processes[8]. For example, deterministic processes play a larger role than stochasticity in driving the fungal community assembly in wild stonefly. Conversely, some studies suggest that stochastic processes such as drift and dispersal limitation have a greater impact on host community assembly[8]. Recent research on the gut microbiota of *Apis cerana*, *Apis mellifera*, *Drosophila simulans and Dicranocephalus wallichii bowringi* has indicated that stochastic processes like dispersal limitation and drift are the key factors in shaping gut microbiota assembly[8,9]. These species-specific differences not only demonstrate a significant role of the insect host in the assembly of its symbiotic community but also underscore the influence of different environments and ecological niches on the structuring process of symbiotic bacteria.

Insect pests can develop resistance to xenobiotics, including chemical insecticides, over prolonged exposure. Despite restrictions on pesticide use in some countries to mitigate insecticide resistance and environmental risks, insect pests continue to evolve, leading to escalating levels of resistance in recent years[10]. Host-microbe interactions can mediate insecticide resistance, reflecting the coordinated response of the host and its microbiome to insecticides[11]. Insect pests with varying insecticide resistance levels often exhibit distinct microbiome structures, diversity and functions[12,13]. However, existing studies have primarily focused on core microorganisms associated with insecticide resistance, overlooking the impact of symbiont interactions on the insecticide resistance in their hosts[14]. The collective response of the host and its microbiome to insecticides may manifest in differences in community assembly, influencing host evolution in response to insecticides[15]. However, the impact of insecticide exposure on host microbiota assembly and its subsequent feedback on host phenotypes requires further investigation.

The primary question revolves around whether insecticide stress influences the selection of the host and its symbionts. If yes, how much such influences may lead to changes in microbiota assembly processes and potentially impact the host's insecticide-resistant phenotype. Addressing the above-mentioned critical questions is challenging due to the multifaceted interactions in complex natural environments. To overcome this challenge, we designed a series of experiments to explore the causal relationship between insecticide exposure, community assembly processes of host symbionts, and the resulting insecticide resistance phenotypes in a laboratory model insect pest, i.e., *Spodoptera frugiperda* or the fall armyworm, a globally invasive notorious insect pest in agriculture. We hypothesize that insecticide exposure induces alterations in the symbiotic community assembly of insect pests, leading to changes in microbial interactions that subsequently impact the host's insecticide resistance phenotype. By integrating microbiome analysis, community assembly modeling, and insecticide resistance evolution analysis, we confirmed the hypothesis that insecticide exposure significantly modifies the community assembly processes of host symbiotic bacteria, transitioning between deterministic and stochastic processes based on exposure duration. This shift in community assembly processes was found to delay the evolution rate and heritability of insecticide resistance in the insect pest host. This study underscores the pivotal role of symbionts in shaping the evolution of insect pest insecticide resistance and offers a foundational basis for developing symbiont-targeted insect pest control strategies.

Materials and methods

Field sampling and laboratory experimentation of insects

The six field populations of *S. frugiperda* were collected from maize fields in five provinces of China, including Zhejiang, Guangdong, Anhui, Hubei and Guangxi in 2022 (Table S1). Fifth-instar larvae and their faeces were collected on-site, and the larvae were immediately dissected to obtain gut tissue and contents. All field populations were brought back to the laboratory to assess their resistance levels to three insecticides including, chlorantraniliprole, indoxacarb and lambda-cyhalothrin. NN population was randomly chosen for long-term indoor feeding for future experiments, such as short-term insecticide exposure and insecticide resistance evolution assay. The larvae were fed an artificial diet in the laboratory following a previous study[16]. Adults were housed in 30 cm × 30 cm × 30 cm insect cages and provided with a 10% honey solution for nutritional supplementation. All insects were maintained at 25 ± 1 °C and 65 ± 5% relative humidity (RH) under 16-hour light:8-hour dark (L:D) photoperiod.

The indoor-resistant populations were selected from a field population (SS) collected from maize in Sanya, Hainan Province (109.12°E, 18.37°N), during the 2021 crop season. The selection bioassays were conducted using third-instar larvae of the F2 generation. The resistant populations were named chlorantraniliprole-resistant population (CR), indoxacarb-resistant population (IR) and lambda-cyhalothrin-resistant population (LR), respectively.

Three chemical insecticides used in this study (chlorantraniliprole 98%, lambda-cyhalothrin 96% and indoxacarb 95%) were provided by ShanDong WeiFang Rainbow Chemical Co., Ltd and HuBei WeiDeLi Chemical Technology Co., Ltd, respectively. Stock solutions of the insecticides were diluted with acetone or *N*, *N*-dimethyl formamide to the desired concentrations.

Short- and long-term insecticide exposure of *S. frugiperda*

The diet-overlay method was used for field population insecticide bioassays as described previously with a slight modification[17,18]. In detial, 900 μL of artificial diet was added to a well in 24-well plates. Subsequently, 100 μL of the insecticide solution was added to the diet surface to ensure even coverage. Five concentrations of insecticide (prepared by diluenting with distilled water containing 0.1% Triton X-100) and a control (0.1% Triton X-100 in distilled water) were used for bioassay for *S. frugiperda*. After the insecticide solution had dried, 24 healthy 3rd-instar larvae of *S. frugiperda* were carefully placed individually in each well of the plates, with three replicates for each concentration. The treated insect larvae were maintained in the insect-rearing environment and mortality was recorded at 24 h (lambda-cyhalothrin), 48 h (indoxacarb and chlorantraniliprole) after treatment. The lethal concentration of 50% (LC_{50}) of *S. frugiperda* to each insecticide was calculated using Polo Plus (ProbitLogitAnalysis, Le-OraSoftware).

The NN population was treated with sub-lethal concentrations of insecticides (LC_{50} of each insecticide) for 24 h, representing short-term insecticide exposure populations (chlorantraniliprole, indoxacarb and lambda-cyhalothrin exposure named C, I and L, respectively) (Fig. 1a). The SS population was treated with sub-lethal concentrations of insecticides (LC_{50} of each insecticide every generation) for 48 h each generation and maintained for 10 (IR) or 15 (CR and LR) generations as a long-term insecticide exposure population (Fig. 1a).

DNA extraction, 16S rRNA gene amplicon sequencing and bioinformatics analysis

Total genomic DNA of insecticide-treated populations of *S. frugiperda* was extracted using the OMEGA Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions. The extracted DNAs were quantified and their quality was assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

PCR amplification of the bacterial 16S rRNA gene V3–V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGG CAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTC TAAT-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components included5 μL of buffer (5×), 0.25 μL of Fast pfu DNA Polymerase (5 U/μL), 2 μL (2.5 mM) of dNTPs, 1 μL (10 μM) of each forward and reverse primer, 1 μL of DNA template, and 14.75 μL of ddH_2O. Thermal cycling consisted of initial denaturation at 98 °C

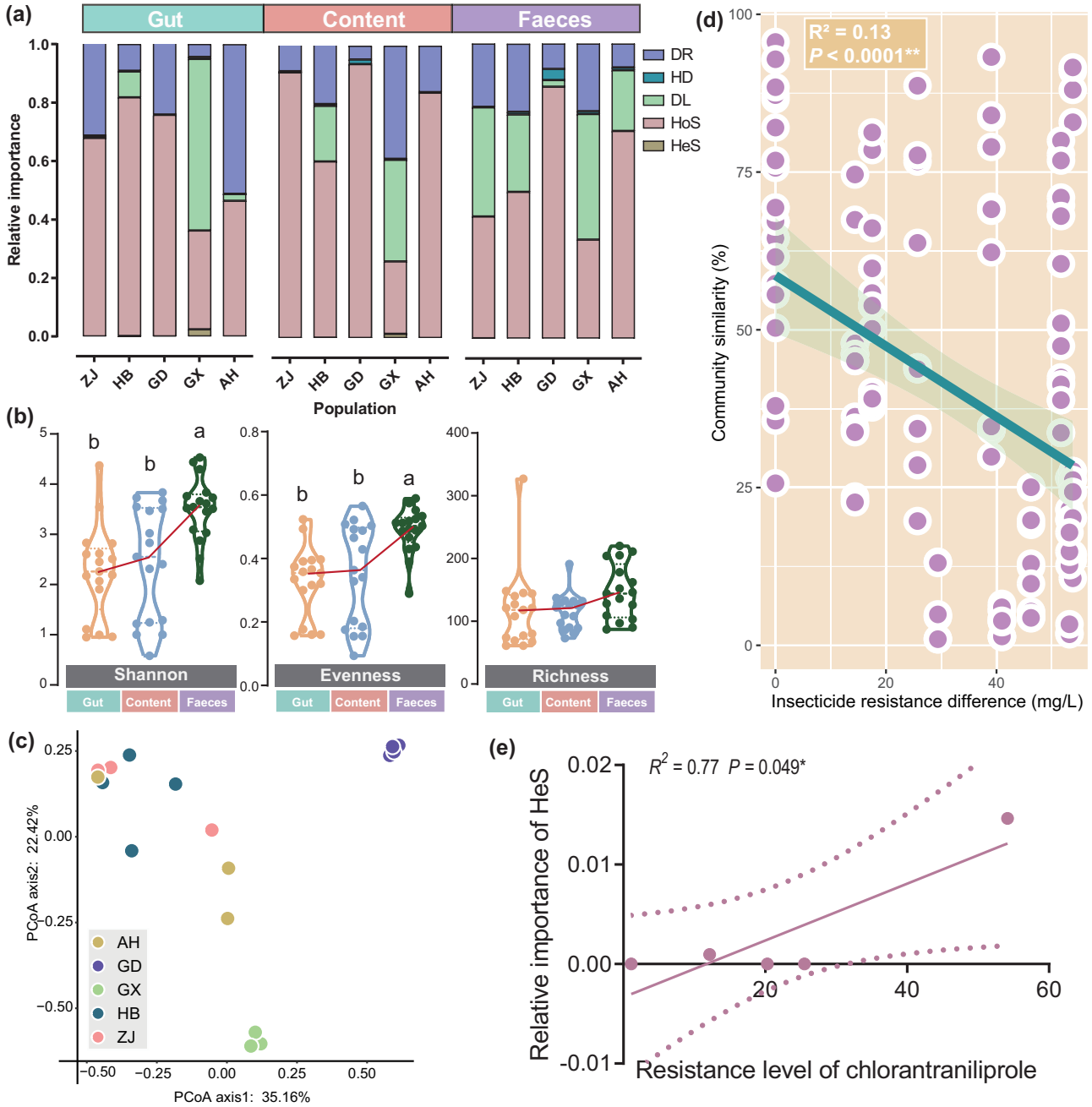


Fig. 1 | The relationship between microbiome diversity, community assembly mechanisms and their relation to insecticide resistance levels in field fall armyworms. a Relative importance of microbiota assembly processes in the gut, gut content and faeces of 5 strains of field fall armyworms. **b** The α-diversity (Shannon, Evenness and Richness) of the microbiome in the gut, gut content and faeces of 5 strains of field fall armyworms, $n = 17$ biologically independent samples. **c** PCoA analysis of the microbiome of the gut, gut content and faeces of 5 strains of field fall armyworm. **d** Correlation analysis of microbiome similarity and differences in insecticide resistance in gut contents of 5 strains of field fall armyworms. **e** Correlation between the relative importance of heterogeneous selection (Hes) and the resistance level of fall armyworms to chlorantraniliprole, $n = 5$ biologically independent strains. DR: Drift; HD: Homogeneous diffusion; DL: Diffusional limitation; HoS: Homogeneous selection; HeS: Heterogeneous selection. "*" and "**" represent significantly difference set up as $P < 0.05$ and $P < 0.01$, respectively.

for 5 min, followed by 25 cycles of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 5 min at 72 °C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After individual quantification, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

The 16S rRNA gene amplicon sequence data were analyzed using QIIME2 The data were demultiplexed with the demux plugin and then subjected to primer cutting with the cutadapt plugin[19]. subsequently, the sequence data underwent processing with the DADA2 plugin for quality filtering, denoising, merging, and removal of chimeras. Non-singleton amplicon sequence variants (ASVs) were aligned using maft. Taxonomy was assigned to the ASVs using the classify-sklearn naïve Bayes taxonomy classifier in the feature-classifier plugin, against the Silva138 Database.

Analysis of community assembly processes of *S. frugiperda* symbionts under insecticide exposure

The contribution of different ecological processes to community assembly based on phylogenetic bin-based null model analysis was identified using MbioAssy2.0 (https://github.com/emblab-westlake/MbioAssy)[20] which implements the iCAMP package[6]. To quantify various ecological processes, the observed taxa are initially categorized into distinct groups, referred to as 'bins', based on their phylogenetic relationships. Subsequently, the processes governing each bin are identified through null model analyses of phylogenetic diversity, utilizing the beta Net Relatedness Index (βNRI), as well as taxonomic β-diversities assessed via the modified Raup–Crick metric (RC). For each bin, the proportion of pairwise comparisons exhibiting βNRI values less than -1.96 is interpreted as the percentage of homogeneous selection, while those with βNRI values exceeding +1.96 are regarded as the percentage of heterogeneous selection, in accordance with previously established thresholds. Following this, the taxonomic diversity metric RC is employed to analyze the remaining pairwise comparisons where |βNRI| is less than or equal to 1.96. The fraction of pairwise comparisons with RC values below -0.95 is classified as the percentage of homogenizing dispersal, whereas those with RC values above +0.95 are categorized as indicative of dispersal limitation. The remaining comparisons that fall within the ranges of $|\beta NRI| \leq 1.96$ and $|RC| \leq 0.95$ are interpreted as representing percentages of drift, diversification, weak selection, and/or weak dispersal[6].The observed taxa were first divided into different 'bins' according to a phylogenetic signal threshold ($ds = 0.2$, bin.size.limit = 12) within the phylogenetic tree to determine the relative importance of heterogeneous selection, homogeneous selection, homogenizing dispersal, dispersal limitation.

Co-occurrence network based on the Spearman's rank correlation was created using the "Co-occurrence_network.R" script in MbioAssy2.0 package. A threshold of Benjamini-Hochberg method-adjusted $P < 0.05$ and $r > 0.6$ was subsequently applied to eliminate weak interactions[7,20]. Global network properties were analyzed using the Molecular Ecological Network Analysis Pipeline[21,22] and visualized with Gephi v0.9.2.

Evolution risk assessment of insecticide resistance and estimation of realized heritability

Wild-type insects were fed an artificial diet with or without antibiotics (1000 mg/L ciprofloxacin and 1000 mg/L rifampicin) continuously. Subsequently, the insects were subjected to insecticide resistance selection. Each generation was screened using the LC_{50} of the previous generation and mortality rates were recorded.

The realized heritability (h^2) of resistance to chlorantraniliprole in *S. frugiperda* and antibiotic-treated *S. frugiperda* was estimated using a threshold trait analysis method, as described by Tabashnik[23,24]:

$$h^2 = R/S \tag{1}$$

where R is the response to selection and S is the selection differential.

The response to selection (R) is the difference in mean phenotype between the offspring of the selected parents and the entire parental generation before selection, and it was estimated as:

$$R = \frac{\log(final\ LC_{50}) - \log(initial\ LC_{50})}{n} \tag{2}$$

where the final LC_{50} is the LC_{50} of offspring of the insect surviving after "n" generations of selection, the initial LC_{50} is the LC_{50} of the parental generation before "n" generations of selection and R is the average response to selection per generation.

The selection differential (S) represents the difference in mean phenotype between the selected parents and the entire parental generation, which was estimated as:

$$S = i\sigma_p \tag{3}$$

where i is the intensity of selection and σ_p is the phenotypic standard deviation.

The intensity of selection (i) was estimated from p, the percentage of surviving selection, which is based on the properties of a normal distribution. The phenotypic standard deviation was estimated as the reciprocal of the mean of the estimated slopes of the probit regression lines from the selected colony (σ_p = 1/average slope).

The response to selection (R) can be estimated as the product of heritability (h^2) and selection differential (S):

$$R = h^2 S \tag{4}$$

Based on the response of insects to selection in the laboratory, the number of generations required for a tenfold increase in LC_{50} (G) is the reciprocal of R:

$$G = R^{-1} = (h^2 S)^{-1} \tag{5}$$

h^2 was used to predict the rate of insecticide resistance development at different mortality rates, representing the strength of selection), with mortality rates of 50%, 60%, 70%, 80%, 90% and 99%, respectively. Assuming the initial and final slope of estimated slopes of the probit regression lines were the same (b = 2), the generations required (N) for resistance evolution to increase by100 times (RR = 100) at each selection strength, as follows:

$$N = \frac{(b_1 + b_2) \times \log(RR)}{2h^2 i} \tag{6}$$

Statistics and reproducibility

The source data for this study are stored in database Figshare[25], identified by the doi numbers of https://doi.org/10.6084/m9.figshare.26867302 (Fig. 1), https://doi.org/10.6084/m9.figshare.26868445 (Fig. 2), https://doi.org/10.6084/m9.figshare.26868451 (Fig. 3), https://doi.org/10.6084/

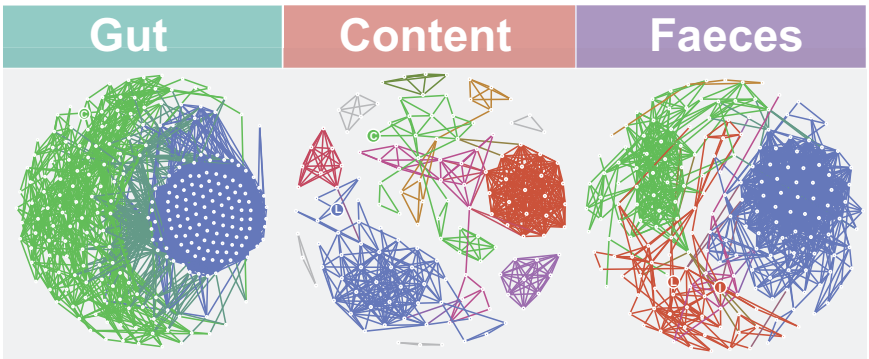
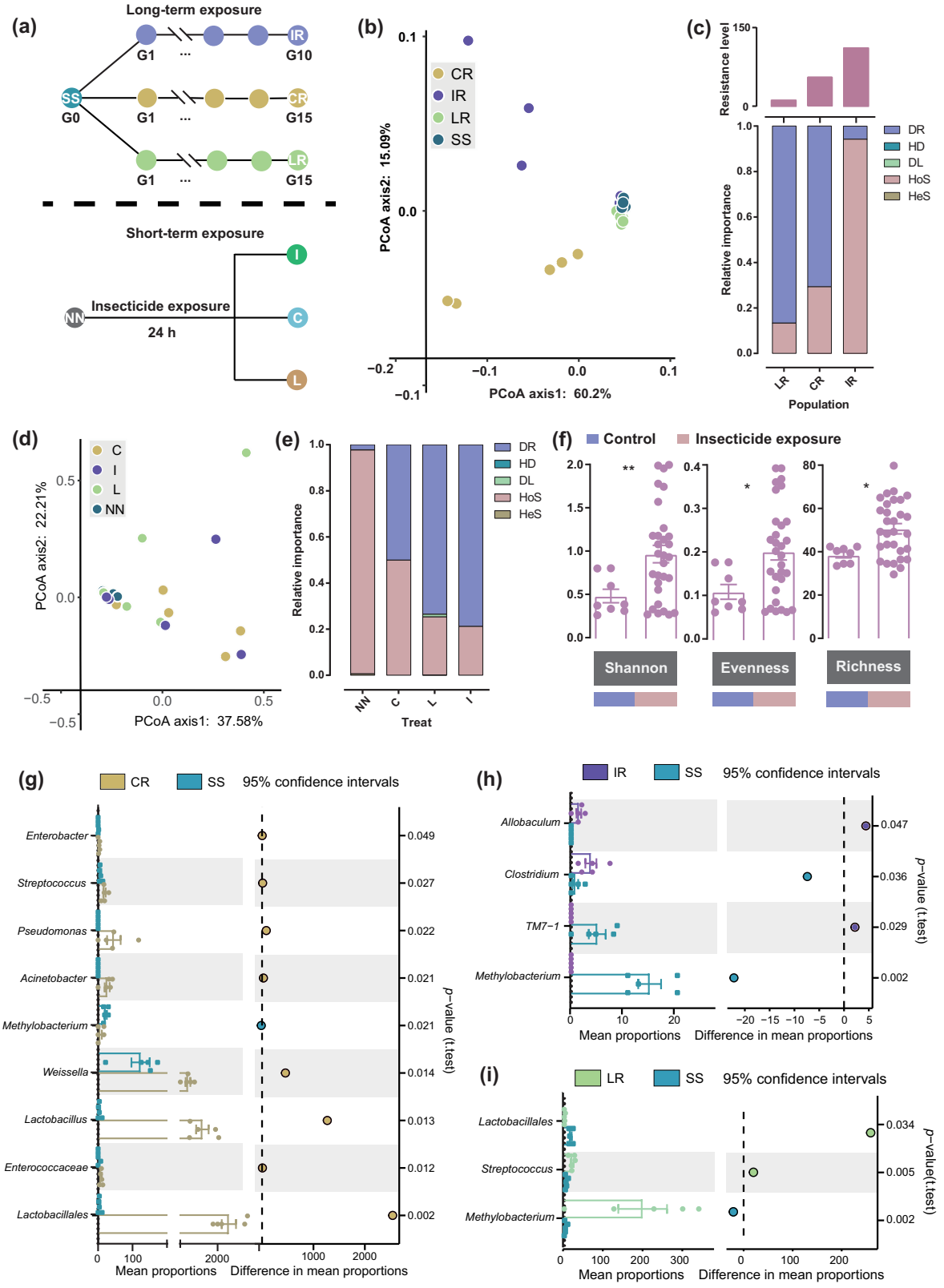


Fig. 2 | Co-occurrence networks showing community-wide correlation between insecticide resistance and symbiont abundance in the field fall armyworm. Three networks were created to illustrate the correlation between bacteria abundance in the gut, gut content and faeces samples and insect's insecticide resistance. The colors represent the different modules highlighting ecological niches. Details of the network analysis are available in Table S3.



m9.figshare.26868475 (Fig. 4) and https://doi.org/10.6084/m9.figshare.26868481 (Fig. 5).

The α-diversity and β-diversity of insect microbiota were estimated using the diversity plugin of q2-diversity with rarefied samples, including three α-diversity indices [Shannon, evenness, and richness]. The VEGAN package (version 2.5-7) was used to calculate the Bray–Curtis distances between samples with the ASVs table, and principal coordinate analysis (PCoA) was performed with distance matrices using the cmdscale function in R. All results from R were visualized and plotted using ggplot2 (version 3.3.3). The microbiome function prediction was performed by PICRUST2 (version 2.5.2). The bacterial taxa and their prediction function difference analysis of different populations or treatments were performed with

Fig. 3 | Long- and short-term insecticide exposure shaped the symbiont community assembly process and diversity in fall armyworm. a Experimental design. **b** PCoA analysis of microbiome in insecticide resistant (long-term insecticide exposure) and susceptible strains of fall armyworm. **c** Relative importance of symbiont community assembly process and, respectively, resistance level in three insecticide resistant fall armyworm strains. **d** PCoA analysis of fall armyworm microbiome in short-term insecticide exposure. **e** Relative importance of symbiont community assembly process of fall armyworm microbiome in short-term insecticide exposure. **f** The α-diversity (Shannon, Evenness and Richness) of microbiome of insecticide exposure, $n = 9$ and 30 for control and insecticide exposure, respectively, biologically independent samples, the bar chart is shown as mean ± standard error. **g–i** Differences in symbiotic abundance between resistant and susceptible fall armyworm strains, $n = 5$ biologically independent samples, the bar chart is shown as mean ± standard error. SS: susceptible strain; IR: Indoxacarb-resistant strain; CR: chlorantraniliprole-resistant strain; LR: lambda-cyhalothrin-resistant strain. NN: Nanning strains; I: Indoxacarb exposure; C: chlorantraniliprole exposure; L: lambda-cyhalothrin exposure. DR: Drift; HD: Homogeneous diffusion; DL: Diffusional limitation; HoS: Homogeneous selection; HeS: heterogeneous selection. "*" and "**" represent significantly difference set up as $P < 0.05$ and $P < 0.01$, respectively.

STAMP analysis on the online Omicstudio (https://www.omicstudio.cn/index).

Multiple regression analysis was performed using R packages Car (version 3.1) and MASS (version 7.3). Car package was utilized to test whether the relationship between variables and dependent variables satisfies the model conditions (including linear relationship, data independence, normal distribution, residual, etc.), and then to obtain the optimal model equation using the "backward" method of the MASS package.

Significance analysis was performed by *t*-test (for two groups) and multiple comparison (for multiple groups) using GraphPad 8, with "*", "**" and different letters indicating significant differences ($P < 0.05$).

Results

The entire microbiome collectively shapes the field insecticide resistance of *S. frugiperda*

Insecticide resistance facilitated by gut microbes raised global concerns on its threats on agricultural health and sustainability. In order to explore whether the insecticide resistance level of *S. frugiperda* was related to the symbiont, we collected 5 field populations from different provinces of China and measured the LC_{50} of these populations to three insecticides. The experimental results showed that compared with the susceptible strain (SS), the resistance level of the field *S. frugiperda* collected was distributed from low-level resistance to high-level resistance to chlorantraniliprole (1.12-53.89), lambda-cyhalothrin (0.41-1.57) and indoxacarb (48.59-119.76) (Table 1). Based on the variation in insecticide resistance levels of the field population, we hypothesize that the variation in insecticide resistance levels among field populations of *S. frugiperda* may be influenced by the effects of insecticide exposure, leading to alterations in symbiont community assembly processes. To investigate this, we collected gut, gut content, and feces samples from in-situ larvae in the field for 16S rRNA gene amplicon sequencing. Subsequently, we calculated the relative importance of the main processes of symbiont community assembly.

The results revealed that homogeneous selection (24.76–90.87%), dispersal limitation (0–58.66%) and drift (4.74–51.19%%) were the predominant processes, collectively explaining 96.87% to 99.96% of community assemblage (Fig. 1a). Similar to the variation in insecticide resistance, the symbiont community assembly process varied among field populations. For instance, the GX population was primarily influenced by dispersal limitation (DL) process, while the ZJ, HB and GD populations were mainly driven by homogeneous selection (Hos) (Fig. 1a). Additionally, we observed that the α-diversity (Shannon, evenness, and richness) increased in the order of gut > content > faeces, correlating with their increasing levels of environmental exposure (Fig. 1b). The relative importance of the symbiotic assembly process in three out of five populations (ZJ, HB and AH) increased in diffusional limitation process with the extent of environmental exposure (Fig. 1a). Among these populations enriched in dispersal limitation process, we noted that the symbiont composition was more similar, while GX and GD exhibited more variation compared to those three populations (Fig. 1c). Another explanation for this noticeable difference is geographical dependent, as the similarity in gut microbiome structure between populations decreases with geographic distance (Fig. S1). According to the Mantel test, the gut symbiont community showed a strong correlation with the insecticide resistance of insect (Table S2). The differentiation in insecticide resistance also significantly correlated with the similarity in gut symbiont community, suggesting that insecticide application not only enhances insecticide resistance but also influences the symbiont community of *S. frugiperda* (Fig. 1d). Importantly, the relative proportion of heterogeneous selection in the deterministic processes was significantly positively correlated with the resistance level of chlorantraniliprole ($P = 0.049$, Fig. 1e). These findings indicate that the gut microbiome is closely associated with the insecticide resistance levels of *S. frugiperda* under complex field environmental exposure.

We further sought to identify the core symbionts associated with insecticide resistance in field *S. frugiperda*. The correlation-based association network between symbiont abundance and insecticide resistance level were analyzed. We identified three patterns of resistance level-symbionts and symbionts-symbionts networks in different positions of the microbiome (Fig. 2). In contrast to the intensive correlation among symbionts-symbionts (average degree: 55.79, 7.12 and 12.91 in gut, content and faeces, respectively), resistance level-symbionts showed weak correlation: only 4 (3 correlated with chlorantraniliprole and 1 correlated with indoxacarb, average degree: 1.33), 5 (2 correlated with chlorantraniliprole and 3 correlated with indoxacarb, average degree:1.67) and 13 (9 correlated with indoxacarb and 4 correlated with lambda-cyhalothrin, average degree: 4.33) bacterial taxa were associated with insecticide resistance of insect in gut, content and faeces, respectively (Fig. 2). However, 7383, 595 and 1198 pairwise associations between bacteria taxa were identified in the gut, content and faeces, respectively (Fig. 2). The number of significant bacterial associations in the gut was significantly higher than in the contents and feces, suggesting a high degree of reciprocal cooperation among gut symbionts (Fig. 2 and Table S3).

Based on topological analysis and modularity (md) comparison between the observed co-occurrence networks (Table S3), we found clear patterns of niche convergence (i.e., one major module) in the insect gut microbiome while niche differentiation or segregation (i.e., multiple modules) in the contents and faece microbiomes (Fig. 2). There are 9.25, 2.00, and 3.15 average correlation connections (i.e., edges) with insecticide resistance-related nodes, which are significantly lower than the average degree (ad: 55.79, 7.12 and 12.91) in gut, content and faeces microbiota, respectively (Fig. 2 and Table S3). We found that the clustering coefficient (cc) of the networks decreased in the following order: gut (0.90) > content (0.76) > faeces (0.69), suggesting environmental factors exposure loosens the aggregation patterns of microbiota community (Fig. 2 and Table S3). We also analyzed the contribution of different microbes to insecticide resistance using multiple regression analysis. The results indicated that the abundance of 6 out of the top 10 genera significantly explained the insecticide resistance level by 24% to 77% (Table S4). Among them, *Enterococcus*, *Microbacterium* and *Methylobacterium* were the more important predictors on insecticide resistance of insect (Table S4). Insecticide resistance of fall armyworm can be affected by a maximum of one to four microbials, while only *Methylobacterium* in faeces, alone was predicted to significantly affect the resistance to chlorantraniliprole (Table S4). These results strongly imply that the symbiont variation caused by insecticide exposure may depend on the interaction between the symbiont species, whereas the contribution of individual bacteria is weak.

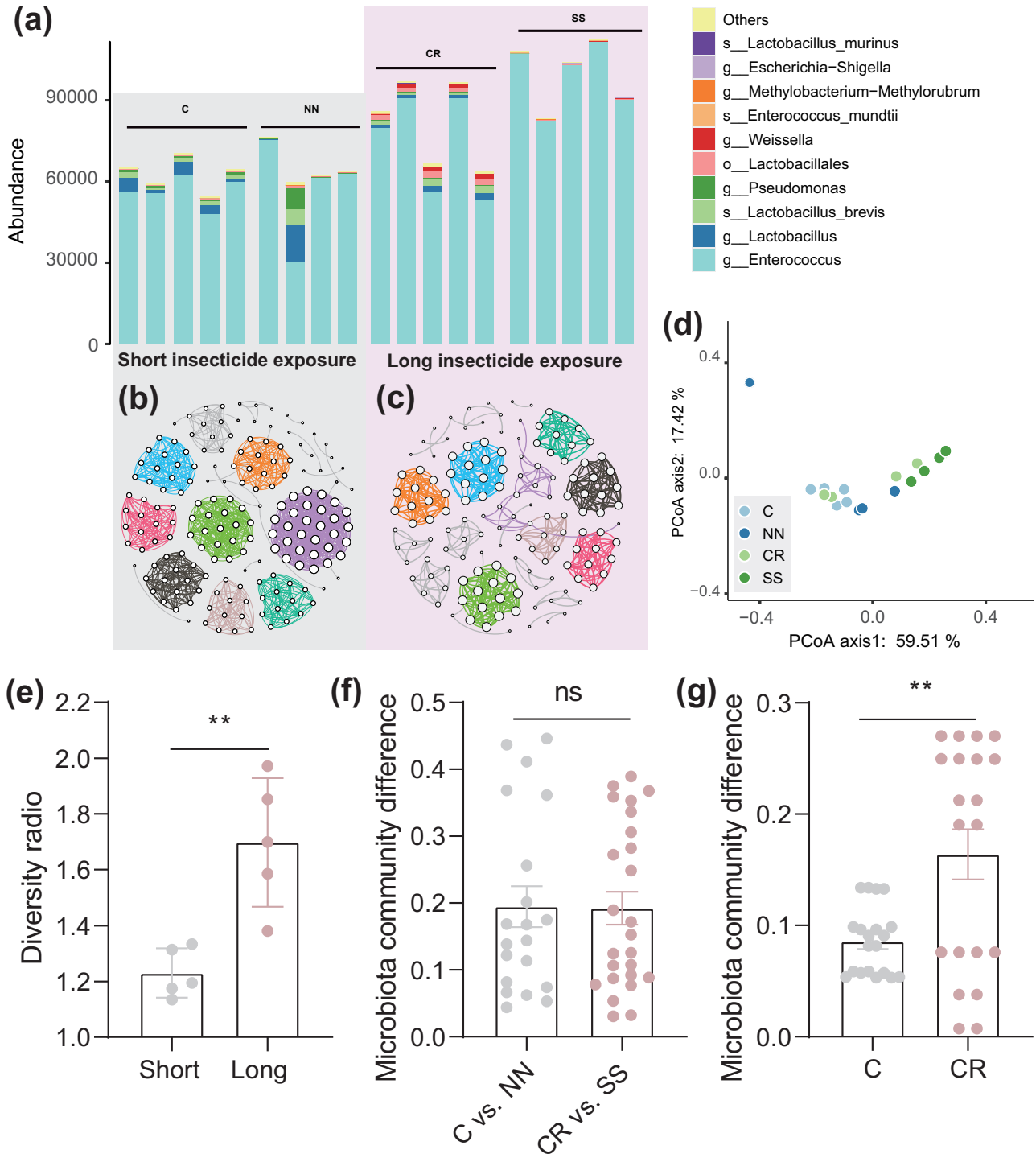


Fig. 4 | Differences in the impacts of long-term and short-term insecticide exposure on host microbial communities. a Abundance of the top 10 microbials before and after long-term and short-term insecticide exposure. **b**, **c** Co-occurrence networks between microbial taxa in short-term (**b**) and long-term (**c**) insecticide exposure. The colors represent the different aggregation modules of species, highlighting their ecological niches. Details of the network can be found in Table S5. **d** PCoA analysis of the fall armyworm microbiome in long-term and short-term insecticide exposure. **e** α-diversity ratio of long-term and short-term insecticide exposure over their respectively initial strains, $n = 5$ biologically independent samples, the bar chart is shown as mean ± standard error. **f**, **g** Microbiota difference of long-term and short-term insecticide exposure over their respectively initial strains (**f**); and differences in microbiota within populations after insecticide exposure (**g**), $n = 20$ biologically independent samples, the bar chart is shown as mean ± standard error. "*" and "**" represent significantly difference set up as $P < 0.05$ and $P < 0.01$, respectively.

long-term and short-term insecticide exposure altered the diversity of symbionts and the processes of community assembly

We further hypothesize that short- and long-term exposure to insecticides not only induces a stress response in the *S. frugiperda* host, but also affects its bacterial symbiont. Thus, 16S rRNA gene amplicon sequencing was used to analyze the response of symbionts to insecticide exposure (Fig. 3a). After the susceptible strain (SS) was exposed to three commonly used insecticides in the field for multiple generations, the resistance of the insect host to

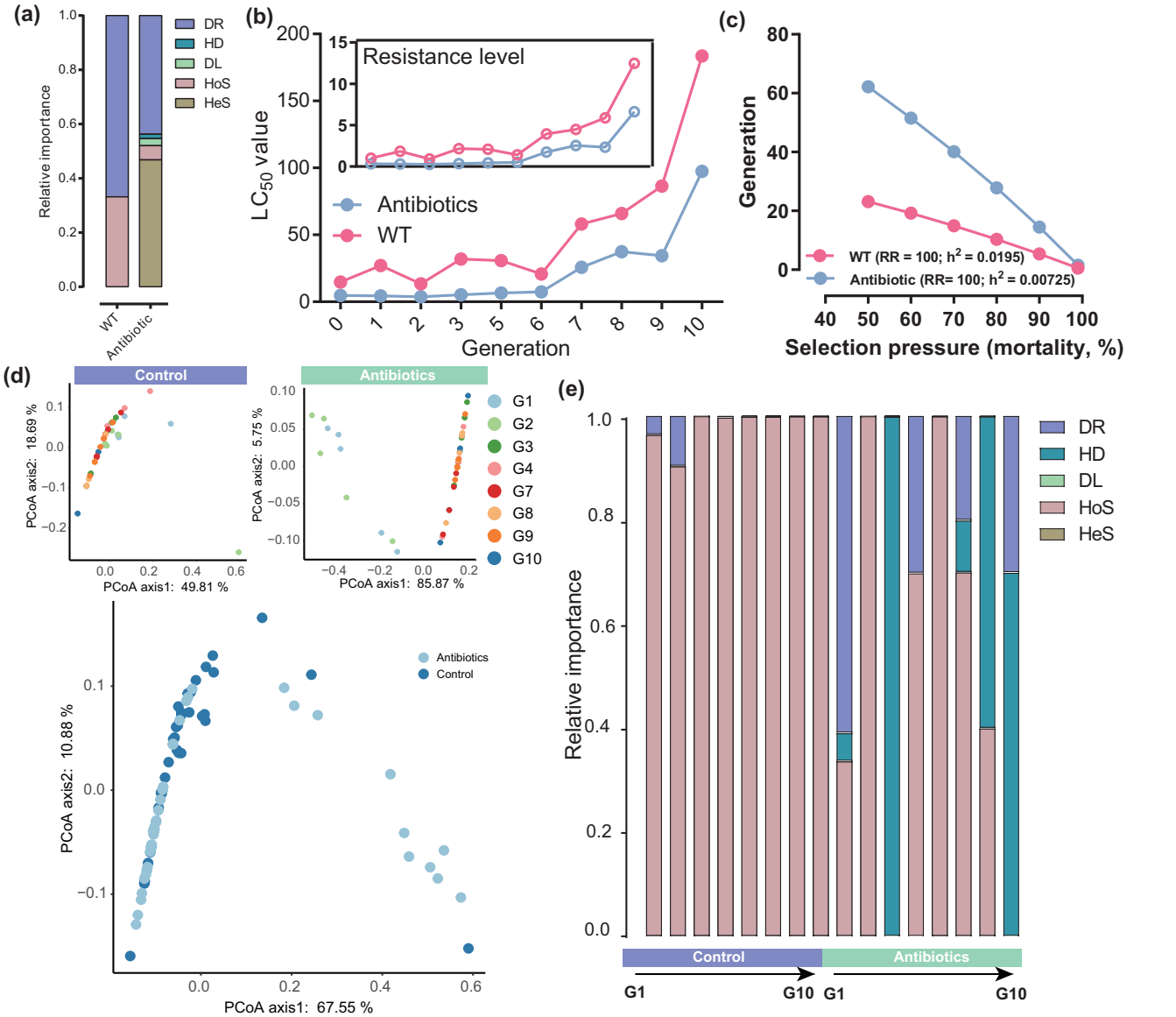


Fig. 5 | The establishment of symbiotic community of fall armyworms disrupted feedback on the evolution of the host's resistance to chlorantraniliprole. a The influence of antibiotics on the symbiont community assembly process of fall armyworm. **b** Insecticide evolution of fall armyworms to chlorantraniliporle. **c** Insecticide resistance heritability and the predicted rate of insecticide resistance evolution. **d** PCoA analysis of fall armyworm microbiome in insecticide resistance evolution between control and antibiotic-treated strains. **e** Relative importance of the symbiont community assembly process of fall armyworms across insecticide evolution. DR: Drift; HD: Homogeneous diffusion; DL: Diffusional limitation; HoS: Homogeneous selection; HeS: Heterogeneous selection.

chlorantraniliprole (CR), lambda-cyhalothrin (LR) and indoxacarb (IR) significantly increased by 10.77, 55.14, and 110.57 times, respectively (Table 1). The symbionts' β diversity of SS and the three resistant populations was not significantly different (Fig. 3b, PERMANOVA: $R^2 = 0.046$, $P = 0.94$). The relative importance of the five ecological processes of microbiota assembly, i.e., homogeneous selection (HoS), heterogeneous selection (HeS) homogeneous dispersal (HD), drift and others (DR) and dispersal limitation (DL), was different among the three resistant populations, reflected in the relative importance of HoS increasing with the host's resistance level to insecticide (Fig. 3c), which implies insecticide resistance development may promote the dominant process from stochasticity to deterministic of the host's symbiont community assembly. Short-term exposure to these insecticides had a weak effect on the symbionts β diversity (Fig. 3d, PERMANOVA: $R^2 = 0.15$, $P = 0.62$), whereas the relative importance of symbiont community assembly processes changed, increasing in the DR process with decreasing HoS compared to the control (NN), which implies that short-term insecticide exposure makes the symbiont community assembly process become stochastic (Fig. 3e). There are different patterns of symbiont community assembly between long- and short-term insecticide exposure, the diversity, richness and evenness increased after the insecticide exposure (Fig. 3f). Among them, the relative abundance of rare species (relative abundance < 0.5%) in resistant strains (CR, LR and IR) increased compared to the SS strain, including *Lactobacillus*, *Weissella*, *Lactobacillales*, *Allobaculum* and *Streptococcus* (Fig. 3g–i). These results indicate that insecticide exposure changed the symbiont diversity and community assembly pattern of *S. frugiperda* and distinguished by the duration of insecticide exposure.

Different responses of insect gut microbiota in long-term and short-term insecticide exposure

We found that chlorantraniliprole exposure exhibited the strongest and effects on the host microbiome, leading to differences in microbial diversity and composition (Fig. 3b, g). Therefore, we further selected this insecticide to resolve and distinguish between short-term and long-term exposure

Table 1 | LC_{50} of *S. frugiperda* population to insecticides used in this study

Insecticide	Population	N	Slope (SE)	LC_{50} (95%CI)	χ^2 (df)	RR
Chlorantraniliprole	AH	144	1.60 (0.38)	1.01 (0.28-1.74)	0.77 (3)	1.12
	GX	144	1.18 (0.26)	48.50 (26.88-166.13)	1.62 (4)	53.89
	GD	144	2.07 (0.26)	18.19 (13.41-25.08)	4.14 (4)	20.21
	HB	144	1.15 (0.13)	18.80 (9.92-17.05)	1.46 (4)	20.89
	ZJ	144	1.53 (0.34)	22.85 (14.36-59.26)	2.04 (3)	25.39
	SS	168	2.66 (0.45)	0.90 (0.69-1.31)	1.18 (4)	1.00
	CR	180	2.32 (0.35)	49.38 (38.63-67.46)	3.29 (3)	54.87
	NN	144	1.19 (0.27)	14.7 (7.70-60.86)	4.25 (4)	16.33
Lambda-cyhalothrin	AH	144	1.57 (0.31)	19.88 (13.87-30.76)	1.69 (3)	0.45
	GX	144	2.24 (0.43)	43.81 (30.02-89.63)	0.66 (3)	0.99
	GD	144	2.15 (0.28)	69.4 (52.59-103.41)	1.08 (4)	1.57
	HB	144	2.06 (0.24)	18.28 (13.81-21.04)	2.94 (4)	0.41
	ZJ	144	2.36 (0.40)	30.84 (22.97-48.80)	1.78 (3)	0.70
	SS	252	2.94 (0.35)	44.14 (37.60-52.68)	4.88 (4)	1.00
	LR	168	3.51 (0.50)	475.56 (396.05-597.9)	1.83 (4)	10.77
	NN	144	2.07 (0.38)	18.28 (10.26-25.72)	2.71 (3)	0.41
Indoxacarb	AH	144	2.68 (0.40)	8.26 (6.46-10.61)	4.62 (3)	48.59
	GX	144	2.08 (0.37)	16.09 (11.64-27.05)	4.72 (3)	95.82
	GD	144	1.81 (0.22)	20.39 (14.66-27.03)	3.99 (3)	119.76
	HB	144	1.81 (0.22)	18.59 (13.72-23.92)	3.05 (4)	109.35
	ZJ	144	2.15 (0.38)	16.24 (11.81-27.15)	5.37 (3)	95.53
	SS	168	2.54 (0.36)	0.17 (0.13-0.22)	1.71 (4)	1.00
	IR	168	2.40 (0.33)	19.31 (15.03-26.03)	1.74 (4)	113.59
	NN	144	1.76 (0.33)	14.10 (10.02-23.74)	1.12 (4)	82.94

RR indicated Resistance ratio compared to SS.

effects of fall armyworms. The results showed that not only the insect gut microbiota composition differed under both short-term and long-term insecticide exposure, but also their interaction and aggregation patterns showed significant variations ($P < 0.05$, Fig. 4a–c). Short-term stress exhibited higher collinearity network strength (graph density: 0.082 and 0.052 for short- and long-insecticide exposure, respectively), as also evident in the increased number of nodes and edges in its network (short-term exposure: 1207 edges with 172 nodes; long-term exposure: 530 edges with 143 nodes; Fig. 4b, c and Table S5). Conversely, long-term exposure resulted in the fall armyworm's microbial community co-occurrence network exhibiting slightly higher modularity (short-term exposure: 0.81 vs. long-term exposure: 0.89), indicating that long-term insecticide stress enhanced niche differentiation within the insect intestinal microbiota (Fig. 4b, c, Table S5). The average degree of these two co-occurrence networks differed by approximately 2 times (short-term exposure: 14.03 vs. long-term exposure: 7.41), suggesting more frequently and probably more complex interactions among microbes in the short-term insecticide exposure scenario (Fig. 4b, c, Table S5). Besides interaction intensity, there was a significant differentiation in the microbiota structure between fall armyworms exposed to long-term and short-term insecticide exposure ($R^2 = 0.49$, $P = 0.008$; Fig. 4d). Moreover, more pronounced fluctuations in alpha diversity were observed in fall armyworms under long-term insecticide exposure (short-term exposure: 1.23 vs. long-term exposure: 1.70, Fig. 4e). Additionally, although there was no significant difference in the variation of microbial populations with or without insecticide exposure (for both long- and short-term insecticide exposure), the variation within the populations under different treatments significantly increased during long-term exposure, indicating that long-term exposure amplified individual microbiome differences (Fig. 4f–g). Therefore, we further examined the possible changes in microbiome function after insecticide exposure using functional profile of microbiome predicted using PICRUST2. Interestingly, short-term insecticide exposure had a minimal impact on microbiome functional profile, with only 47 differential functional categories identified, most of whose abundance decreased (by 66.00%) after insecticide exposure. In contrast, long-term insecticide exposure revealed 2430 differential functional categories compared to initial populations (SS), with 81.40% showing potential increased functional categories (Supplementary Data 1). Among these increasing potential functional categories, enzymes like cytochrome c oxidase, N-acetyltransferase, and alkane 1-monooxygenase may contribute to insecticide degradation, suggesting that long-term exposure, rather than short-term exposure, may altered the function of the host microbiome by enriching functional pathways involved in insecticide degradation, thereby enhancing insect resistance to insecticides (Supplementary Data 1). Although there are constraints on functional predictions derived from species annotations using PICRUST2, we believe that there are likely functional differences resulting from these changes in microbial community.

Antibiotics enhance the deterministic process of symbiont community assembly and thereupon delayed the evolution of host resistance to insecticides

Based on the significant time-dependent effects we observed of insecticide exposure on the host microbiota. We hypothesize that other strong selective pressures, such as antibiotics, can have a significant impact on the host microbiome, disrupting the shaping process of host-microbe interactions by insecticides and affecting the evolution of resistance to insecticides in the holobiont. After antibiotic treatment, deterministic processes in the microbiota assembly were strengthened, indicating a strong selective pressure of antibiotics on the host gut microbiome (Fig. 5a). Subsequently, an insecticide resistance evolution assay was conducted. The LC_{50} of wild-type

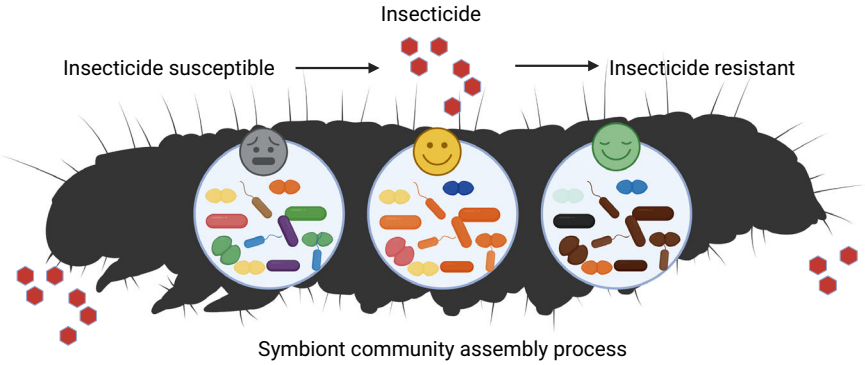


Fig. 6 | Insecticide exposure influenced the microbiota assembly and their insect host, shifting from susceptibility to resistance. The schematic diagram was generated by Biorender (https://app.biorender.com/).

(WT) strains was notably higher than that of the antibiotic-treated strain, resulting in increased resistance levels in WT strains compared to antibiotic-treated strains (Fig. 5b and Table S5). The high heritability of resistance in WT strains, compared to that in antibiotic-treated strains, indicates that more generations are needed to evolve the same level of resistance in antibiotic-treated strains under the same selection pressure. For instance, at a screening mortality rate of 50%, it only takes 23.1 generations to evolve 100 times resistance in WT strains whereas it takes at least 62.2 generations for antibiotic-treated strains (Fig. 5c). We observed that only the first few generations (1-3 generations) of insecticide exposure had a greater impact on the fall armyworm microbiome compared to generation 0 of the WT strain, especially after pre-exposure to antibiotics (Fig. 5d). However, there was no difference in microbiome composition between antibiotic-treated and control strain fall armyworm (Fig. 5d, PERMANOVA: $P > 0.05$). The variation in the relative importance of the microbiota assembly process differed between the antibiotic-treated strain and the control strain of fall armyworm, and also varied between short-term and long-term insecticide exposure (Fig. 5e). In WT strains, insecticide exposure reduced the relative importance of random processes in the host microbiota assembly, while it increased the relative contribution of deterministic processes extent of insecticide exposure. In contrast, the intervention of antibiotics disrupts the gut microbiota assembly process of WT strains, making the assembly patterns of microbiota unpredictable (Fig. 5e). These results indicate that disrupting the homeostasis of the symbiont alters the assembly process of the host symbiont community, further influencing the response of the symbiont community to insecticide exposure, thereby impacting the host's resistance phenotype to insecticides (Fig. 6).

Discussion

The insect host and its symbionts usually face the selection pressure of toxic substances, such as insecticides and plant secondary metabolites[26]. In this study, we investigated the response of insect symbionts to long- and short-term insecticide stress in a global invasive insect pest, the fall armyworm, and explored the consequences of this response for the insect's insecticide resistance. We found that both long-term and short-term insecticide exposure reshaped the microbiota assembly process of the symbiont. Notably, antibiotic-dependent disruption of the gut microbiota alters the host's response and evolution to insecticides. This suggests that the long-term selective pressure of insecticides on the host and symbionts reshapes the symbiotic community and ultimately feedback into the host's resistance phenotype. The results highlight the significant contribution of gut symbionts to insecticide stress adaptation in an invasive agricultural insect pest.

We demonstrated that microbiota interactions contribute much more to the host's insecticide resistance phenotype than individual microbes in the field fall armyworm. The correlation between individual bacterial abundances and the host's LC_{50} values is very weak. Although some studies have identified a number of bacterial isolates that contribute to host resistance[12], it is still important not to ignore the role of microbial interactions in symbiotic function. Such interactions may significantly amplify the contribution of the microbiome to host phenotypic plasticity, thus having a far greater than additive effect on insecticide resistance of insects[27,28]. Interestingly, a previous study demonstrated that the genetic fidelity of the microbiome is a crucial predictor of host phenotypic variation. High genetic fidelity indicates low phenotypic variation[29]. Therefore, simpler symbiotic interactions may enhance the genetic fidelity of the host microbiome, thereby delaying host phenotypic variation. This finding supports the ecological view that high diversity is more stable[16]. Coincidentally, we also observed an increase in microbiota diversity after insecticide exposure, suggesting the positive evolution of the microbiome under insecticide pressure selection. While we cannot definitively say that the changes in species composition are beneficial, we are observing an increase in potential functional differences following prolonged pesticide exposure, suggesting a transformation in microbial function. However, we recognize the constraints of predicting species functions using PICRUST2[30]. To confirm these functional variations, it would be beneficial to utilize more sophisticated third-generation sequencing or metagenomic methods.

Based on the observed differences in the microbiota of insecticide-resistant versus susceptible insect populations, some studies have targeted many insecticide-resistance-associated symbionts, including *Burkholderia*, *Wolbachia*, *Citrobacter sp*., and *Enterococcus casseliflavus*[13,31,32]. However, these studies tend to focus on the consequences of insecticide resistance evolution, ignoring the change in the microbiome and its community assembly process during resistance development. Insecticide exposure significantly reshaped the gut microbiome of fall armyworms, causing a chain reaction of changes in community diversity, assembly patterns, as well as bacterial abundance. It is speculated that the microbiota may be subject to co-selection processes of the host and insecticide based on the differences in the face of long- and short-term exposure. Homogeneous selection process was strengthened with insecticide resistance rising, indicating that increased resistance may lead to greater host selection of the microbiota, whereas short-term insecticide exposure increases the role of drift in microbiota assembly. This finding suggests that insecticide stress disrupts the balanced selection of the host and their gut microbiota, thereby promoting the randomization of the microbiome. Based on this clarification, the evolution of host-microbiome co-resistance may be a process of breaking, reshaping, and breaking again, until the host and microbiome fit into higher concentrations of insecticides.

Determining the factors that impact the rate of insect pest insecticide resistance evolution is a fascinating question. Elucidating this mechanism is expected to address the long-term implications of green insect pest control[33]. We have demonstrated from a unique perspective that microbiota assembly in the insect host can be an effective influencing factor in the evolution of insecticide resistance. The intervention of antibiotics disrupts the host-microbiome balance, leading to the breaking-reshaping and breaking-again process, thus altering the insect's insecticide resistance phenotype variation. However, due to the bacterial resistance to antibiotics is evolving much faster than the host's insecticide resistance, the microbiome variation we observed at a later stage was not significant[34,35]. In fact, we observed very little microbiome variation in subsequent generations except in the first one. The

relative importance of the microbiota assembly process strongly supports the continued effects of antibiotic perturbations on bacterial symbionts. These results imply that a combination of antibiotics and insecticides used in the field may help delay insecticide resistance of insect pests[36].

Based on the microbiome and the interactions within it contribute significantly to insecticide resistance in insects, making it possible to reduce pests' tolerance to insecticides by regulating the abundance of key microorganisms and their interactions. Various biological and physical methods, such as phage-targeted cleavage of insect probiotics, using nanotransducer-based precise control of engineered bacteria to suppress the pathogens in the useful insect gut, and other genetic techniques, can modify the assembly of the insect microbiota to make it toward human-friendly with beneficial or harmful phenotypes they mediated of their insect hosts[32,37]. However, the practical application of these approaches in agricultural settings dealing with insect pests remains need further clarification. While previous studies have reported how symbiosis in some insects may influence the evolution of host insecticide resistance, the role of microbial interactions has not received much research attention and can be further exploited[32]. In particular, there is still a lack of in-depth mechanistic understanding of how microbial interactions impact the insect host's phenotypic characteristics such as regulation of host signaling pathways by cross-feeding products produced by microbial cooperation. Future research utilizing meta-omics approaches, such as metagenomics, metatranscriptomics, and metabolomics, to comprehensively analyze the microbial community taxonomy, functional potential, and metabolic activities should greatly offer novel insights into these intestinal microbial interaction traits underlying host stress resistance.

In summary, we found that the time-dependent response to insecticide by the symbionts of an important invasive insect pest as a whole, rather than some individual symbiosis, may be a potential driving force for the evolution of insecticide resistance in both laboratory and field strains of fall armyworm. Breaking the rule of this response reduces the heritability of host insecticide evolution. Future research should focus on the interactions between those symbionts that play more critical roles in the insecticide resistance evolution of fall armyworms. This study will help to understand the variation mechanism of insecticide resistance evolution and provide a theoretical basis for symbionts-targeted insect pest control.

Data availability

The sequencing raw data generated in this study are deposited in the China National GeneBank DataBase under the accession numbers of CNP0004265 (short-term exposure experiments) and CNP0005629 (field strains, long-term exposure and insecticide evolution experiments).

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Author contributions

Conceptualization: Y. Zhang, F. Ju. Methodology: Y. Zhang, K, Mao, Z. Zhao, F. Ju. Investigation: Y. Zhang, K, Mao, K. Chen. Visualization: Y. Zhang. Supervision & funding: F. Ju. Writing—original draft: Y. Zhang. Writing—review & editing: F. Ju, Y. Zhang, K, Mao, Z. Zhao, K. Chen.

Competing interests

The authors declare no competing interests.

Additional information

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