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Gut symbiotic bacteria enhance reproduction in *Spodoptera frugiperda* (J.E. Smith) by regulating juvenile hormone III and 20-hydroxyecdysone pathways

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Abstract

Background The insect gut microbiota forms a complex, multifunctional system that significantly affects phenotypic traits linked to environmental adaptation. Strong reproductive potential underpins the migratory success, population growth and destructive impact of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). However, the precise role of gut bacteria in *S. frugiperda* reproductive processes, distribution and transmission dynamics remains unclear.

Results We examined the gut microbiota of *S. frugiperda* a major invasive agricultural pest, identifying *Enterococcus*, *Enterobacter*, and *Klebsiella* as core microorganisms present throughout its life cycle. These microbes showed heightened activity during the egg stage, early larval stages and pre-oviposition period in females. Using an axenic insect re-infection system, *Enterococcus quebecensis* FAW181, *Klebsiella michiganensis* FAW071 and *Enterobacter hormaechei* FAW049 were found to significantly enhance host fecundity, increasing egg production by 62.73%, 59.95%, and 56.71%, respectively. Metagenomic and haemolymph metabolomic analyses revealed a positive correlation between gut symbiotic bacteria and hormone metabolism in female *S. frugiperda*. Further analysis of metabolites in the insect hormone biosynthesis pathway, along with exogenous injection of juvenile hormone III and 20-hydroxyecdysone, revealed that gut microbes regulate these hormones, maintaining levels equivalent to those in control insects. This regulation supports improved fecundity in *S. frugiperda*, aiding rapid colonization and population expansion.

Conclusions These findings emphasize the pivotal role of gut bacteria *E. quebecensis* FAW181, *E. hormaechei* FAW049, and *K. michiganensis* FAW071 in enhancing *S. frugiperda* reproduction by modulating JH III levels through JHAMT regulation and concurrently modulating the levels of 20E and its precursors via PHM. Our results provide novel insights into microbe–host symbiosis and pest management strategies for alien invasive species.

Keywords Fall armyworm, Gut bacteria, Juvenile hormone III, 20-hydroxyecdysone, Fecundity

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Background

Microorganisms commonly colonise animals, aiding the host's adaptation to challenging environments [1, 2]. In insects, hosts provide a stable environment and essential nutrients for microorganisms while the microorganisms participate in key activities, such as nutrient metabolism and detoxification [3–6]. These interactions play crucial roles in host evolution. Long-term symbiosis shapes intestinal homeostasis and markedly influences host nutrition, development, immunity, reproduction and other phenotypes essential for environmental adaptation [2, 7]. For example, yeast-like symbiotic fungi and the symbiotic bacterium *Arsenophonus nilaparvatae* enable the synthesis of essential amino acids and vitamin B in the host *Nilaparvata lugens*, which otherwise lacks these capabilities [8, 9]. Similarly, gut microorganisms can degrade caffeine to reduce its toxicity in *Hypothenemus hampei*, whereas the symbiotic bacterium *Enterobacter* sp. EbPXG5, which colonises the intestinal epithelium of *Plutella xylostella*, protects the gut and degrades secondary metabolites, such as kaempferol, enhancing *P. xylostella*'s adaptability to its host plant [10, 11]. These complex, long-term symbiotic relationships substantially affect host biology, ecology and evolution.

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is an omnivorous insect native to the tropical and subtropical Americas [12]. In recent years, this species has emerged as a globally important invasive agricultural pest [13–15]. Notably, the strong migratory ability and adaptability of *S. frugiperda*'s enable rapid spread, population establishment and severe agricultural damage. Its larvae feed on a wide range of food and cash crops, including maize, rice, wheat and peanuts. In Brazil alone, *S. frugiperda* has been recorded on 353 plant species [16], causing substantial economic losses. The larval gut lepidopteran hosts diverse commensal bacteria, predominantly from the phyla Proteobacteria, Firmicutes and Bacteroidetes [17]. The dominant genera include *Klebsiella, Enterococcus, Acinetobacter, Enterobacter, Sphingobacterium* and *Pseudomonas*, which are associated with digestion and immunity [18, 19]. Beneficial gut microbe–host symbiosis contributes to hosts' extensive host range and environmental adaptability [7]. Maternal transmission of gut microbiota, often mediated by egg surface deposition, establishes essential microbial communities in offspring [20]. Throughout the life cycle of *S. frugiperda*, significant shifts in microbiota composition occur during molting and metamorphosis, yet core bacteria such as *Enterococcus* are conserved, underscoring their vital role in insect development and health [21, 22].

S. frugiperda exhibits high food intake and robust reproductive capacity, with a single female laying up to 1439 eggs [23]. This strong reproductive potential underpins the species' migratory success, population growth and destructive impact. Gut microorganisms influence host health and reproduction. For instance, the intracellular symbiotic bacterium *Wolbachia* can alter insect host reproduction by inducing parthenogenesis, cytoplasmic incompatibility, feminisation or male-killing [24–27]. Extracellular symbionts also affect host reproduction; inhibiting gut bacteria in the olive fruit fly, *Bactrocera oleae*, reduces protein and essential amino acid content, leading to a notable decline in host oviposition [28]. Conversely, introducing *Pseudomonas putida* into the diet enhances oviposition in *B. oleae* [29], whereas *Bactrocera dorsalis* egg production increases markedly when it is fed *Pantoea dispersa* and *Enterobacter cloacae* [30]. *S. frugiperda* reproduction is influenced by various factors, including photoperiod, humidity and nutritional status [31–33], with juvenile hormones (JH) and 20-hydroxyecdysone (20E) playing key regulatory roles. JH and 20E are pivotal hormones in the regulation of vitellogenesis in insects. JH acts as the principal gonadotropin, stimulating vitellogenesis in hemimetabolous and most holometabolous insects, while 20E is essential for vitellogenesis in certain lepidoptera, diptera, and hymenoptera species [34, 35]. Gut dysbiosis affects diverse biological processes in the host [36], implying that gut bacteria play an important role in host adaptation to the environment. However, how gut dysbiosis impacts host reproduction and the underlying mechanisms remain unclear.

To clarify the role of gut bacterial symbiosis in *S. frugiperda*, we analysed the gut bacterial communities and their changes throughout the insect's life cycle. Using an axenic insect-reinfestation system, we investigated the impact of gut bacteria on host reproduction. Mechanisms underlying microbial influence were explored through metagenomic and haemolymph metabolomic analyses. Finally, the role of gut bacteria in host reproduction was verified via JH III and 20E injections. Our findings demonstrate that gut microorganisms influence host reproduction through hormonal pathways, offering novel insights into gut microbe–host symbiosis as well as *S. frugiperda*'s environmental adaptation and population expansion.

Methods

Insect population and sampling

Samples representing different development stages of *S. frugiperda* were collected from Xundian (25°50′34.5′′ N, 103°7′12.2′′ E, Yunnan, China), at the station of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Populations were trapped during the peak migration period using high-altitude searchlights (JLZ1000BT; Shanghai Yaming Lighting Co.,

Ltd., Shanghai, China). Twenty females caught with the searchlights were reared on 5% honey, and their eggs were collected. Larvae were reared across generations on maize leaves and sampled at all developmental stages of F1 generation. Specifically, approximately 200 eggs were collected. Larvae were categorized into young larvae (larvae-Y), comprising 50 first-instar larvae (L1) and 30 second-instar larvae (L2), and older larvae (larvae-O), including 10 third-instar larvae (L3) and 10 fourth-instar larvae (L4). Additionally, five individuals each of fifth-instar larvae (L5) and sixth-instar larvae (L6) were sampled. Pupae and adults were also collected, with 1-day-old males and females (AMD1 and AFD1, respectively) and 5-day-old males and females (AMD5 and AFD5, respectively) sampled at five individuals per sample. For each developmental stage, three to four biological replicates were generated. DNA was subsequently extracted and 16S rRNA sequenced.

An experimental *S. frugiperda* population was collected in 2019 from a cornfield in Dehong Prefecture, Yunnan Province, and reared in the laboratory for more than 20 generations on an artificial diet [37]. The laboratory conditions were maintained at 25 °C ± 1 °C, 70% ± 10% relative humidity and a 16 h light: 8 h dark photoperiod. The experiment involved axenic and bacterial reinfections of *S. frugiperda* using an experimental population.

Axenic insects

The experimental *S. frugiperda* populations were provided water containing 5% honey and antibiotics (300 μg/mL ciprofloxacin hydrochloride and 300 μg/mL streptomycin sulphate). Antibiotic treatments were applied only to the larval stage to avoid effects on adult reproduction. Eggs were collected daily and immersed in 1.5% sodium hypochlorite for 30 s, followed by gentle agitation in 75% anhydrous ethanol for 90 s. Eggs were washed twice with sterile water and air-dried on an ultra-clean workbench [38]. Larvae were hatched in 25-mL sterile plastic cups (38 × 30 × 30 mm) and fed an antibiotic-containing diet, which was replaced every 3 days. Larvae were reared aseptically, and sixth instar larvae were switched to an antibiotic-free diet until pupation. Pupae were transferred to new sterile plastic cups, and emerging adults were used as axenic specimens. For the next generation, the same procedure was repeated, with antibiotics being discontinued at the fourth instar. The fourth instar larvae and adults from this generation were then used in subsequent experiments.

DNA extraction and sequencing

Eggs, first to third instar larvae and pupae were sterilised via immersion in 75% anhydrous ethanol for 3 min, followed by two washes in sterile water, and DNA was extracted directly. Fourth to sixth instar larvae, 1-day-old females, 1-day-old males, 5-day-old females and 5-day-old males were sterilised as described above, followed by gut dissection and DNA extraction, with four replicates per development stage. DNA was extracted using the TIANamp Genomic DNA Kit (PD304; Tengen Biochemical Technology Co., Beijing, China), following the manufacturer's protocol. The V4 region of the 16S rRNA gene was amplified using primers 515F and 806R [39]. The sequencing libraries were prepared using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), validated for quality and sequenced on the NovaSeq6000 platform.

Bioinformatics analyses were performed using QIIME 2 [40]. Effective tags were obtained by applying QIIME's Tags QC process and comparing sequences with species annotation databases to identify and remove chimeric sequences [39, 41]. The raw reads were first quality controlled by fastp (version 0.19.6), filtered and spliced by FLASH (version 1.2.7) to optimize the data, which were processed through the sequential noise reduction method (DADA2/Deblur, etc.) to obtain the ASV (amplicon sequence variant) representative sequence and abundance information. The silva138/16S-bacteria database was used to sort ASVs with 100% sequence similarity as the threshold, followed by further data analyses.

Isolation and identification of culturable bacteria

To minimise contamination from external bacteria, 3-day-old axenic or control females were washed in 75% ethanol for 90 s, followed by two washes in sterile water. Guts were dissected in a 1% NaCl solution, and five guts per sample were homogenised in 1 mL of sterile water in 1.5 mL centrifuge tubes. The homogenate was serially diluted to 10^{-5} and 10^{-6}, and 100 μL of each dilution was plated on LB agar. The plates were incubated at 30 ℃ for 24 h, with five replicates per sample. The number of colony forming units (CFUs) was counted, and the average CFU count per plate was used to calculate the number of CFUs per sample.

Single colonies with distinct morphologies were selected, inoculated in LB medium and cultured overnight at 30 ℃ with shaking at 200 rpm for DNA extraction. DNA was extracted using the TIANamp Genomic DNA Kit (PD302). The 16S rRNA gene was amplified using universal primers 27F and 1492R [39], and the products were sequenced by Sangon Biotech, Inc. (Shanghai, China). The sequences were annotated using the EzBioCloud database [42]. A phylogenetic tree was constructed using representative strains closely related to the isolates, based on 16S rRNA gene data from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Phylogenetic analysis was performed using MEGA 11 with the

maximum likelihood method and 1000 bootstrap replicates [43].

Re-infected live or heat-killed bacteria

To assess the impact of symbiotic bacteria on *S. frugiperda* reproduction, re-infection experiments were conducted with gut bacteria representing > 1% relative abundance (Table S1). The bacterial concentrations were standardised following methods from Behar and Raza [44, 45]. Bacteria were cultured overnight in LB medium, centrifuged, resuspended in phosphate-buffered saline (pH 7) and quantified using OD600. Live and heat-killed bacteria were used for re-infection at a dose of 10^7 CFU/mL. Re-infection of fourth instar larvae and newly emerged adults was performed by adding 20 µL of bacterial suspension to ~ 0.5-cm width cubes of sterile diet, which was replaced every 2 days until pupation. Adults were fed water containing 10^7 CFUs/mL live bacteria via sterile cotton balls for 3 days post-eclosion, with cotton balls changed daily. After 3 days, the adults were fed sterile water until death. Heat-killed bacteria were prepared through autoclaving at 121 °C for 30 min, and axenic insects were treated similarly to obtain heat-killed re-infection samples.

To verify the successful colonisation of the reintroduced bacteria, homogenates of the reinfected adult guts were prepared and plated on LB agar medium. Following incubation, 10 colonies were randomly selected and subjected to 16S rRNA sequencing for identification. The identification results confirmed that all colonies corresponded to the respective reintroduced bacterial species, thereby establishing successful colonisation.

Reproductive parameters and ovarian length measurement

Control, axenic and re-infected adults were paired in ultraviolet-sterilised 500-mL plastic cups covered with sterile gauze and maintained in an aseptic environment. The gauze and cups were replaced daily to collect eggs, with the pre-oviposition period, oviposition period and number of eggs were recorded.

The ovaries were dissected under a stereoscope (SZ380; Shanghai Shangguang New Optical Science and Technology Co., Ltd.) at days 1, 3, 5, 7 and 9 post-eclosion. Ovarian length was measured by photographing the ovaries next to an objective micrometer (C1, DIV = 0.01 mm; Zhuzhou Carter Photoelectric Instrument Co., Ltd.) and analysing the images using ImageJ 1.52a (Wayne Rasband National Institutes of Health, USA).

Metagenomics sequencing

A total 80 individuals 3-day-old axenic and control females were sterilised via immersion in 75% ethanol for 3 min, followed by two washes in sterile water. Guts were dissected, and genomic DNA was extracted using the cetyltrimethylammonium bromide method, with four biological replicates of ten guts per sample. Metagenomic sequencing libraries were prepared using the NEBNext® Ultra DNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's protocol, with index codes added attributing sequences to each sample. DNA was fragmented to ~ 350 bp via sonication, end-polished, A-tailed and ligated with full-length Illumina sequencing adaptors for PCR amplification. PCR-amplified fragments were purified using the AMPure XP system, and libraries were analysed for size distribution on the Agilent2100 Bioanalyzer, followed by quantification using real-time PCR. Subsequently, the libraries were sequenced on an Illumina NovaSeq 6000 platform to generate paired-end reads. Raw data were pre-processed using Readfq (V8) to obtain clean data. Reads were aligned to a host database to exclude host-derived sequences, and the clean reads were assembled using MEGAHIT software (v1.0.4-beta) [46, 47]. Functional annotation was performed using the NCBI database and DIAMOND software (V0.9.9.110) [48, 49].

Haemolymph extraction

Haemolymph was extracted from control, axenic and re-infected adults 3 days post-eclosion. Adults were immersed in 75% ethanol for 3 min with gentle agitation to remove scales, followed by two or three washes in sterile water. After air-drying on an ultra-clean bench, an incision was made on the dorsal thorax of *S. frugiperda* using sterilised Venus clippers. Each adult was placed in a pierced 500-µL centrifuge tube, which was subsequently placed in a 1.5-mL sterile centrifuge tube. The samples were centrifuged at 3000 rpm and 4 °C for 10 min to collect haemolymph in the bottom of the tube. Four biological replicates, each comprising 60 females per treatment, were prepared. The collected haemolymph was stored at − 80 °C for further analysis.

Ultra-high performance liquid chromatography/mass spectrometry assay

Haemolymph samples were diluted (50:200 µL haemolymph: acetonitrile, v/v) to precipitate proteins. Metabolomic analysis was performed using ultra-high performance liquid chromatography (Vanquish UHPLC) coupled with a hybrid quadrupole orbitrap mass spectrometer (Q Exactive™ HF). Ionisation was conducted in positive and negative ion modes. Raw data from the mass spectrometer were processed using Compound Discoverer 3.1 software (CD3.1; ThermoFisher) for peak alignment, peak area extraction and metabolite quantification

[50]. Metabolites were annotated using the KEGG and HMDB databases.

Differentially abundant metabolites were identified using principal component analysis and least squares discriminant analysis with variable projection contribution scores derived from MetaX software [51]. *P*-values were calculated using Student's t-test, and differentially abundant metabolites screening thresholds were set at variable projection contribution > 1, $P < 0.05$ and fold change > 1.5 or < 0.67. Volcano plots visualising differentially abundant metabolites were generated using ggplot2 in R. Normalised metabolite data (z-scores) were used to create clustered heatmaps via the R package Pheatmap.

JH III and 20E injections

JH III (≥ 95%), 20E (≥ 98%), the JH biosynthesis inhibitor precocene II (Pre; ≥ 95%) and the 20E biosynthesis inhibitor azadirachtin (Aza; ≥ 95%) were obtained from Shanghai Yuanye Biotechnology Co. and diluted in DMSO. JH III and 20E were prepared at six concentrations (0, 0.0625, 0.125, 0.25, 0.50 and 1.00 μg/μL) for injection, whereas Pre and Aza were diluted with DMSO to 0.5 μg/μL. Adults were injected with 2 μL of these solutions on the day of eclosion and again on the fourth day post-eclosion.

JH III and 20E assays

JH III and 20E levels were measured using ELISA kits for JH III (YJ077240) and 20E (ML103397) from Shanghai Enzyme-linked Biotechnology Co. Female abdomens were dissected 3 days after injection with 0.5 μg/μL JH III or 20E. The samples were weighed, and 500 μL of ethyl acetate was added. Tissues were thoroughly homogenised and centrifuged at 4000 rpm for 10 min, after which the supernatant was collected in a new centrifuge tube. The extraction was repeated three times, and the combined supernatant was dried using a lyophiliser. Dried residues were dissolved in 100 μL of DMSO for ELISA analysis. Five biological replicates per treatment were prepared. JH III and 20E assays were performed according to the respective ELISA kit instructions.

Data analysis

The data were analysed using SPSS 19 (IBM Corporation, USA), and the results are presented as means ± standard deviations (SDs). Statistical significance was assessed using the Wilcoxon test, Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test for multiple comparisons. Graphs were created using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Network heatmaps were generated using OmicStudio tools (https://www.omicstudio.cn/tool).

Results

Symbiotic bacterial composition of *S. frugiperda* at different developmental stages

To comprehend the dynamics of gut bacterial composition throughout the host life cycle, the microbial profiles of *S. frugiperda* at various developmental stages were analysed (Fig. 1). The results revealed marked shifts in the taxonomic composition of gut bacterial phyla corresponding to the host's developmental stages (Fig. 1A). Principal coordinate analysis showed that the bacterial communities formed distinct clusters for each developmental stage (Fig. 1B, PERMANOVA test, adonis, $R^2 = 0.62$, $P < 0.001$). Specifically, the communities of eggs and young larvae (Larvae-Y, L1 and L2) were closely related, as were those of 5-day-old females and males (AFD5 and AMD5). These groups were distinctly separated from old larvae (Larvae-O, L3-L6), pupae and 1-day-old females and males (AMD1 and AFD1), which formed separate clusters. Old larvae, pupae and 1-day-old adults (AMD1 and AFD1) exhibited higher bacterial richness and greater microbial diversity (Fig. 1C, D). During the egg and early larval stages, the richness (Chao1 index) and diversity (Shannon's index) of gut microbiota were lower, especially in first and second instar larvae. These metrics increased significantly from the third instar larval stage to the pupal stage. In the adult stage, newly eclosed adults (AMD1 and AFD1) displayed the highest richness (Fig. 1C) and diversity (Fig. 1D) indices ($P < 0.05$), which stabilised as the adults aged.

Analysis of bacterial abundance across developmental stages identified Proteobacteria (56.71%) and Firmicutes (27.03%) as the dominant phyla in *S. frugiperda*, followed by Bacteroidota (8.73%) and Actinobacteria (2.61%). At the genus level, the core symbionts that exhibit high abundance and persist throughout the entire life cycle were identified as *Enterococcus*, *Pseudomonas*, *Klebsiella* and *Enterobacter* (Fig. 2A). Dynamic tracking of *Enterococcus*, *Klebsiella* and *Enterobacter* (Fig. 2B–D) revealed their stability throughout the host's life cycle (egg to female adult), beginning in the egg stage. Specifically, the ASV of *Enterococcus* gradually decreased with host age in both larvae and adult females; the ASV of *Klebsiella* was lower in larvae than in eggs, peaked in newly eclosed females and decreased with adult age; and the ASV of *Enterobacter* was highest in eggs, lowest in newly eclosed females and increased with adult age. These findings suggest that gut bacteria play a crucial role in host adaptation during the early larval stages of *S. frugiperda* and may regulate reproduction in the adult stage.

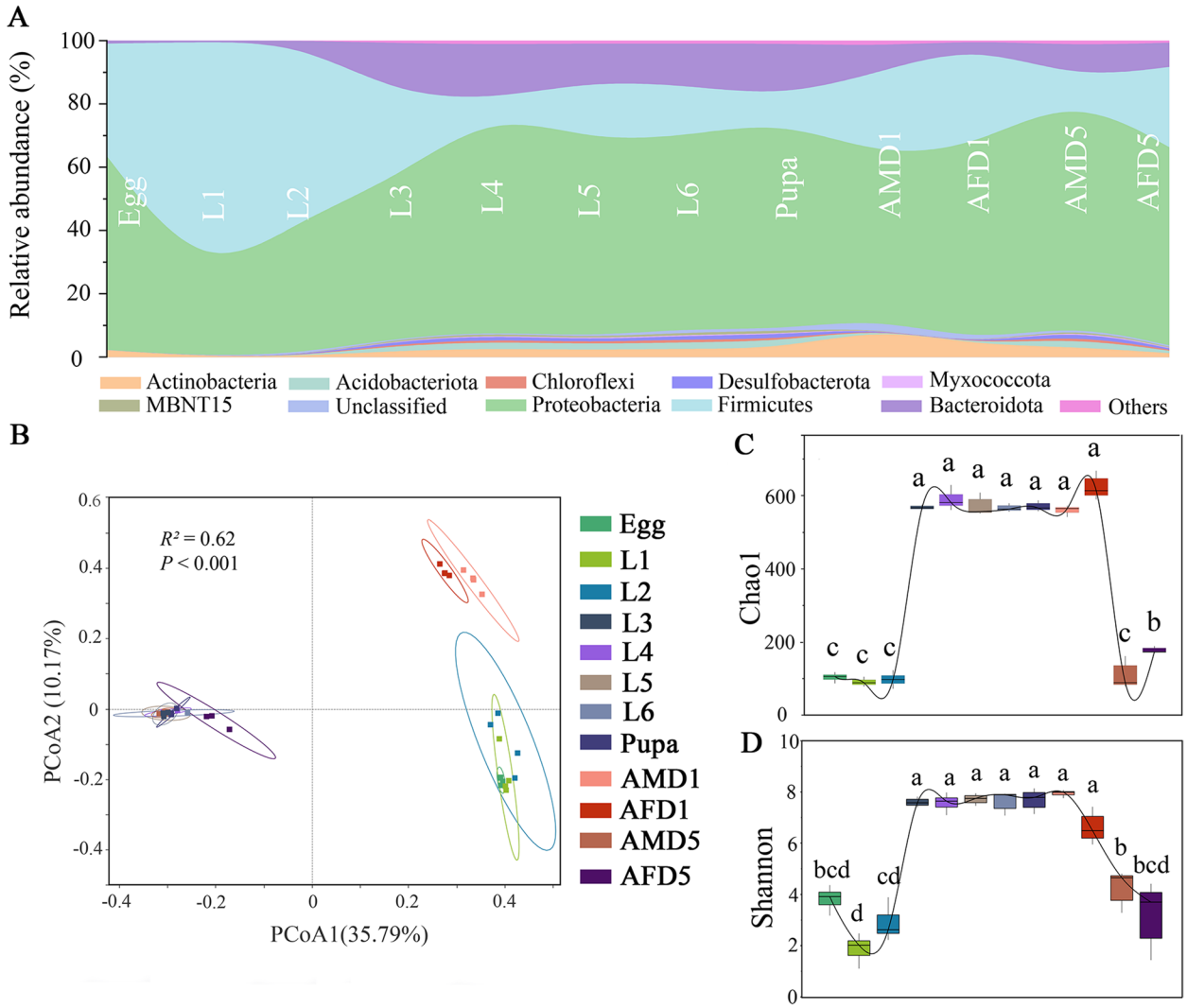

Fig. 1 Microbiota composition of *S. frugiperda* shifts across developmental stages. **A** Relative abundance of bacterial phyla at different developmental stages, visualised using a stream graph. **B** Microbiome compositions across developmental stages represented by Unweighted UniFrac principal coordinate analysis graph. The difference of microbial communities among the developmental stages was calculated with PERMANOVA via "adonis" test. Chao1 (**C**) and Shannon (**D**) indices, with blacke fit lines derived from a generalised additive model, analysed using one-way ANOVA followed by Tukey's multiple comparison test; different letters denote significant differences

Influence of gut bacteria on fecundity in *S. frugiperda*

To determine whether gut bacteria influence reproduction in *S. frugiperda*, antibiotics were used to generate axenic adults. Verification of bacterial removal was achieved by plating gut homogenates on LB agar (Fig. 3A–C) and counting CFUs. Control adults exhibited approximately $4.76 \times 10^7 \pm 1.64 \times 10^7$ CFUs/gut, whereas axenic adults showed no culturable bacteria ($P < 0.0001$; Fig. 3D).

Axenic females laid significantly fewer eggs, with a 43% reduction observed (862 ± 298.77 per female, $df = 105.949$, $P = 0.0001$) compared with control females (Fig. 3E). Egg production also varied depending on male mating partner: axenic females mated with axenic or control males produced significantly fewer eggs, with 46.29% ($P < 0.001$) and 37.31% ($P < 0.001$) reductions observed, respectively (Fig. 3F), compared to control females. Additionally, axenic females had lower mating rates (Fig. S1A). Control females mated with axenic males produced 16.40% fewer eggs ($P < 0.001$; Fig. 3F). Axenic females mated with males exhibited the lowest female longevity (Fig. S1B), although there were no significant differences in male longevity or mating frequency across mating pairs (Fig. S1C, D). Ovary measurements showed that the length of axenic females' ovaries was reduced by 18.82–24.78% ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.01$,

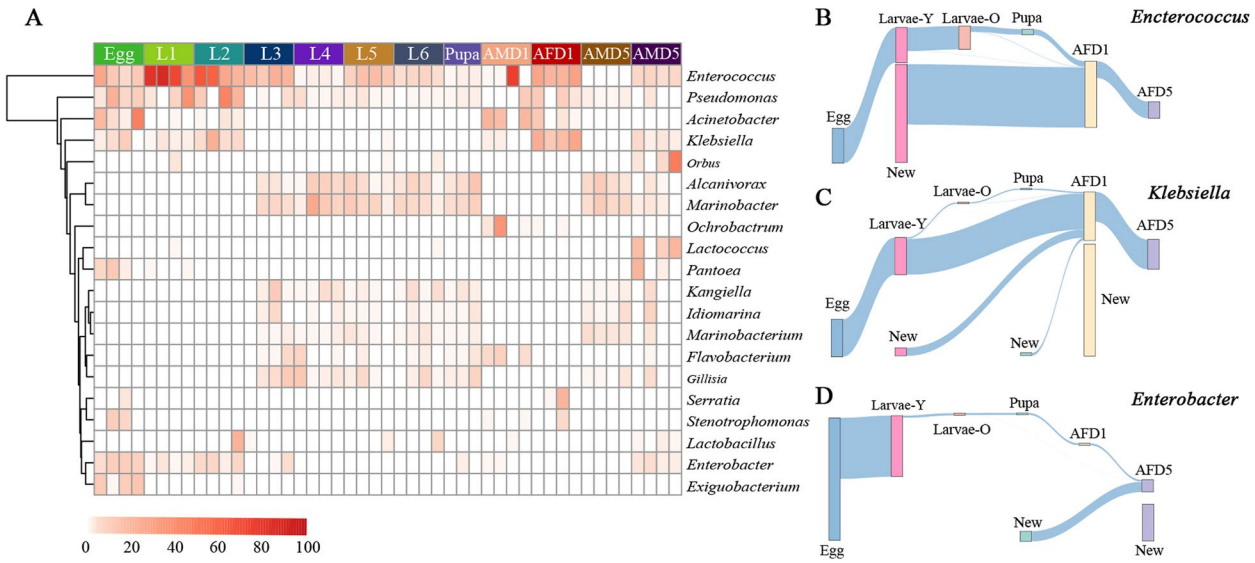

Fig. 2 Major taxa and ASV transferred during *S. frugiperda* development. **A** Heatmap showing dominant bacterial taxa across developmental stages. **B**–**D** Sankey plots tracking ASVs within phylotypes of *Enterococcus* (**B**), *Klebsiella* (**C**) and *Enterobacter* (**D**) throughout development. Rectangle heights indicate relative ASV abundance, Larvae-Y (first and second instar larvae), Larvae-O (third to sixth instar larvae) whereas colours represent developmental stages. Shading illustrates ASV transitions between stages

$P < 0.05$) compared with control females (Fig. 3G). These results demonstrate that gut symbiotic bacteria markedly enhance reproduction in female *S. frugiperda*.

Enterococcus, *Enterobacter* and *Klebsiella* enhance the fecundity of *S. frugiperda*

To identify the gut bacteria influencing reproduction in *S. frugiperda*, we isolated and cultured bacterial strains from adult gut samples and conducted taxonomic identification. In total, 217 bacterial strains were isolated, representing 13 genera and 19 species, as determined through 16S rRNA sequencing. The most abundant strains included *Enterococcus quebecensis* FAW181 (23.50%), *Enterobacter hormaechei* FAW049 (19.35%), *Klebsiella michiganensis* FAW071 (18.89%), *Providencia rettgeri* FAW273 (14.29%) and *Erwinia persicina* FAW223 (7.37%) (Table S1).

To validate the role of these gut bacteria in host reproduction, axenic insects were re-infected with these strains, and their ovarian development and reproductive performance were evaluated. Successful colonization of culturable bacteria in axenic insects was confirmed via 16S rRNA sequencing of colonies from plated gut homogenates (Fig. S2). Re-infection with *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 significantly enhanced ovary development (Fig. 4A–D). Egg production increased significantly in females re-infected with live *E. quebecensis* FAW181 (1301.04 ± 346.32), *E. hormaechei* FAW049 (1278.87 ± 337.92), *K. michiganensis* FAW071 (1252.91 ± 268.84) and *P. rettgeri* FAW273 (1052.96 ± 312.22), representing increases of 62.73%, 59.95%, 56.71% and 31.70%, respectively (all $P < 0.001$), compared with axenic females, although egg production remained lower than that in control adults (Fig. 4F). Conversely, regarding these strains, re-infection with heat-killed bacteria did not significantly improve egg production in axenic adults ($P = 0.99$, $P = 0.51$, $P = 0.38$ and $P = 0.28$, respectively). Notably, there was no significant difference in egg production following re-infection with live and heat-killed *P. rettgeri* FAW273 ($P = 0.26$). Re-infection with a combination of *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 restored egg production in *S. frugiperda* to normal levels (Fig. 4F). Additionally, ovary length, oviposition period and pre-oviposition period were restored to normal levels via re-infection with the three bacterial strains and mixed colonies (Fig. 4G–I). Other bacterial strains, including *Asaia bogorensis* FAW135 ($P = 0.14$), *Leclercia tamurae* FAW119 ($P = 0.20$), *Providencia entomophila* FAW237 ($P = 0.33$), *Pseudomonas* sp. FAW253 ($P = 0.46$), *Bacillus pumilus* FAW154 ($P = 0.66$) and *Erwinia persicina* FAW223 ($P = 0.89$) did not significantly affect egg production compared with that in axenic adults (Fig. S3). These findings highlight the pivotal role of *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 in enhancing fecundity in *S. frugiperda*, with the combined bacterial community playing a critical role in achieving normal reproductive levels.

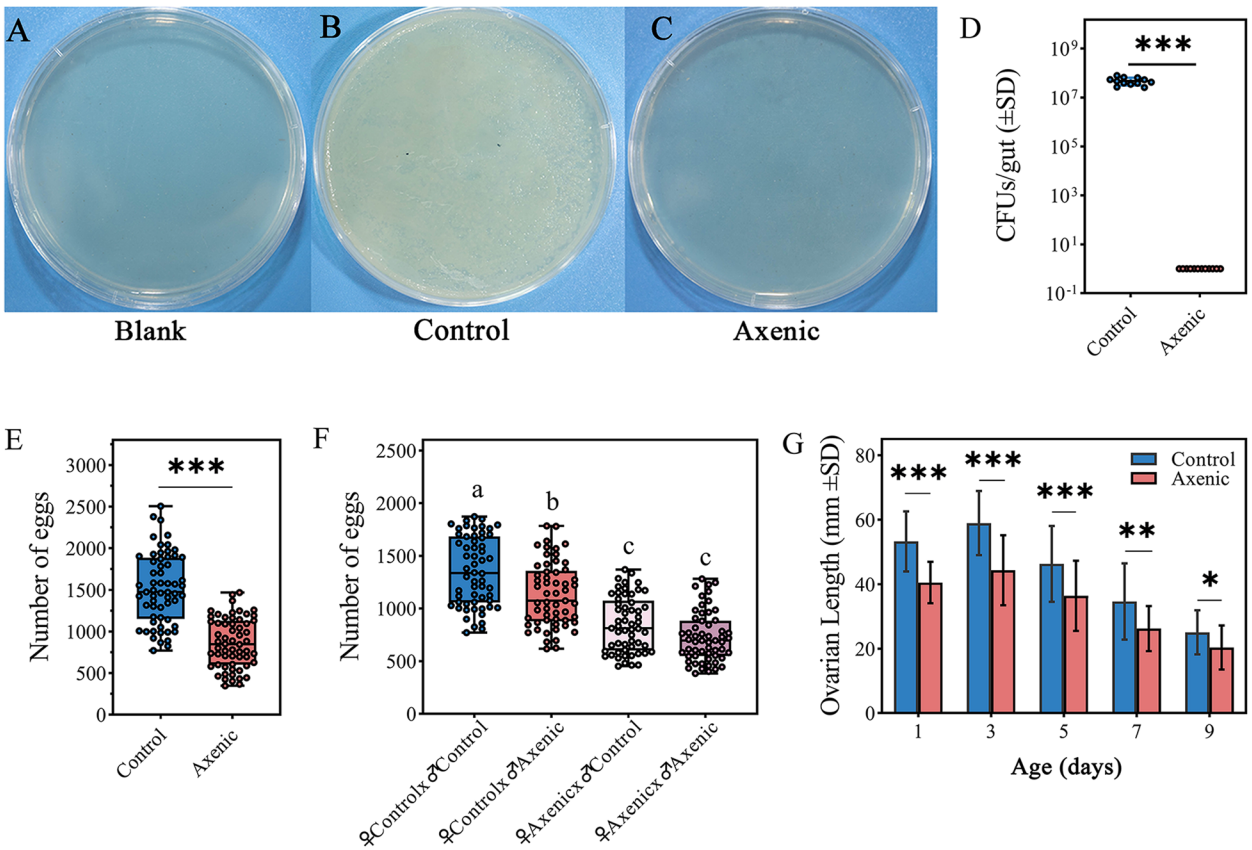

Fig. 3 Influence of gut microorganisms on *S. frugiperda* reproduction. **A**–**D** Verification of microbial elimination in adults through culture of gut homogenates on LB plates: blank control (**A**), control *S. frugiperda* (**B**) and axenic *S. frugiperda* (**C**). (**D**) CFUs of culturable bacteria, analysed using Student's *t*-test ($n = 12$, $^{***}P < 0.001$). **E** Box-and-whisker plots comparing total egg production in control versus axenic adults, analysed via the Wilcoxon test ($n = 60$, $^{***}P < 0.001$). **F** Egg production under different mating patterns ($n = 57$–58), analysed using one-way ANOVA followed by Tukey's multiple comparison test; different letters denote significant differences. **G** Influence of gut bacteria on ovarian development in *S. frugiperda*, analysed via Student's *t*-test, ($n = 28$–36, $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$)

Gut bacteria affect host endocrinology

To explore the mechanisms by which strains FAW181, FAW049 and FAW071 of *E. quebecensis*, *E. hormaechei* and *K. michiganensis*, respectively, promote host reproduction, we compared the metagenomes of axenic and control adults. Additionally, the haemolymph metabolomes of adults re-infected with live and heat-killed bacteria were analysed. Based on 16S rRNA gene analysis, FAW181 was confirmed to be most closely related to *E. quebecensis* CCRI-16985, FAW049 to *E. hormaechei* subsp. *xiangfangensis* and FAW071 to *K. michiganensis* W14 (Fig. 5A).

Metagenomic sequencing analysis results showed that gut microbiome composition differed significantly between axenic and control adults (Fig. S4, Fig. S5A). Notably, *E. quebecensis*, *E. mundtii*, *E. hormaechei* and *K. michiganensis* were absent from the axenic female gut (Fig. S4 and S6A). Additionally, enzymatic pathway analysis of the microbial metabolism pathways linked to insect hormone biosynthesis revealed that JH epoxidase (CYP15A1C1) was enriched in the gut microbiome of control females. In contrast, Juvenile hormone-III synthase (JHAMT), cholesterol 7-desaturase (NVD) and ecdysteroid 25-hydroxylase (PHM) were enriched in axenic females (Fig. S5B, C). These findings suggest that the gut microbiota modulates the expression of enzymes involved in the JH III pathway, specifically by inhibiting the aberrant upregulation of JHAMT to maintain JH III homeostasis, and by suppressing the excessive elevation of NVD and PHM to stabilize the precursor levels of 20E, thereby ensuring 20E homeostasis.

Metabolomic analyses of haemolymph from axenic and re-infected adults detected 1538 metabolites (Table S2). Overall, 131–464 differential metabolites were screened in the haemolymph of axenic females and those re-infected with heat-killed bacteria (*E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071) compared to control females and those

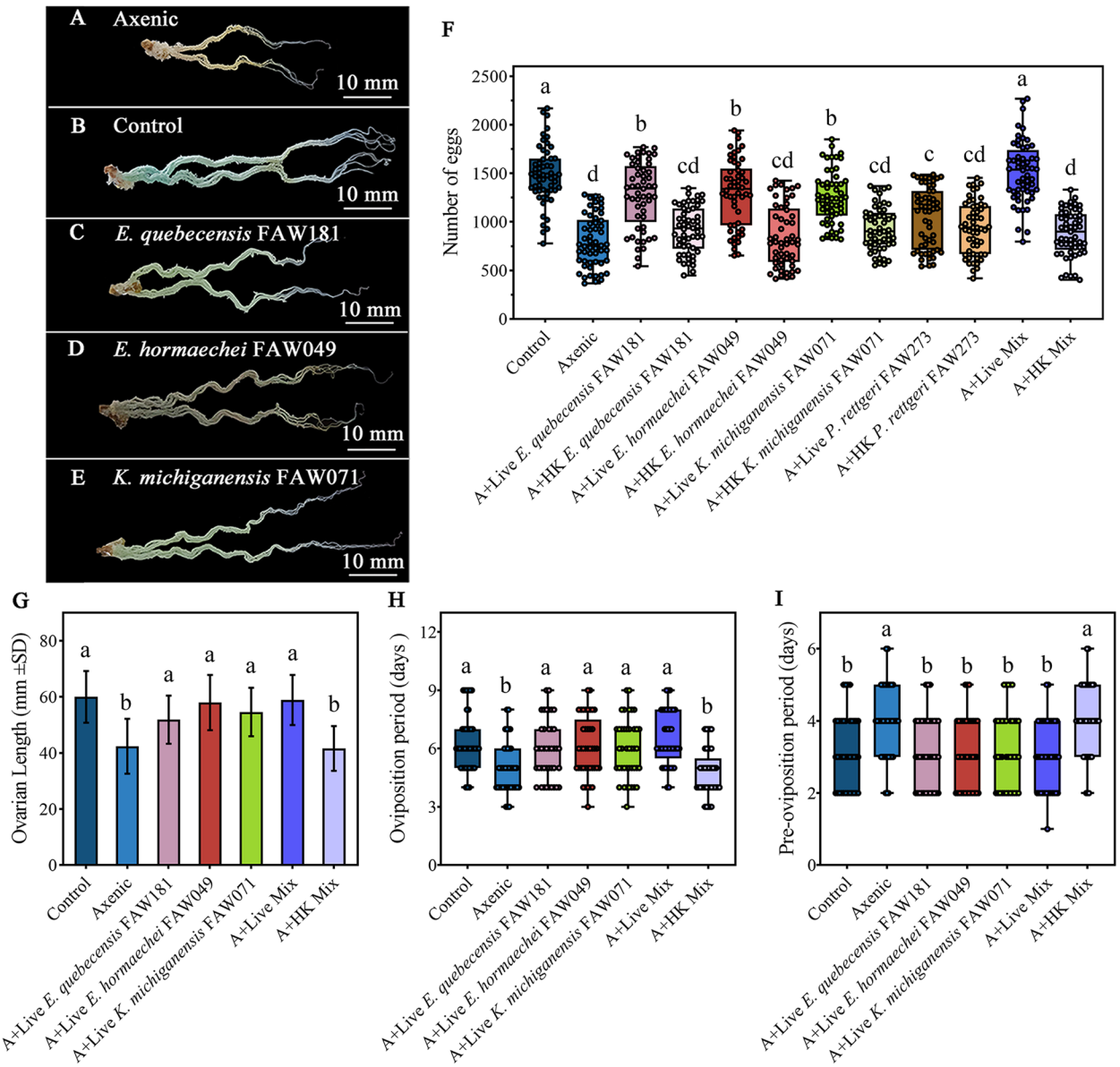

Fig. 4 Effects of re-infection with gut bacteria on *S. frugiperda* reproduction. **A**–**E** Ovaries on day 3 under different treatments: axenic (**A**), control (**B**) and re-infection with *E. quebecensis* FAW181 (**C**), *E. hormaechei* FAW049 (**D**) and *K. michiganensis* FAW071 (**E**). **F**–**I** Comparisons of total egg production (F; $n = 53$–56), ovarian length (G; $n = 34$–39), oviposition period (H; $n = 53$–56) and pre-oviposition period (I; $n = 53$–56) under various treatments, analysed using one-way ANOVA followed by Tukey's multiple comparison test; different letters denote significant differences. Mix: re-infected with *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071

re-infected with live bacteria (Fig. 5C; Table S3), with 40 up-regulated and 16 down-regulated differential metabolites observed across all seven comparisons (Fig. S6B; Table S4). The differential metabolites were primarily categorized into hormones, amino acids and derivatives, and fatty acids and derivatives. Notably, hormones, including 20E, ecdysone and JH III, were predominantly up-regulated in the hemolymph of axenic insects. Combined metagenomic and metabolomic analyses indicated strong positive correlations (Pearson's $R > 0.9$) between the abundance of the gut bacteria *E. quebecensis*, *E. mundtii*, *E. hormaechei* and *K. michiganensis* in *S. frugiperda*, and these bacteria were strongly associated with host membrane transport, metabolism of cofactors and vitamins as well as metabolism of terpenoids and polyketides (Mantel's $R \geq 0.85$, $P < 0.05$; Fig. 5B). Furthermore, KEGG enrichment analysis highlighted the significant involvement of differentially abundant metabolites in

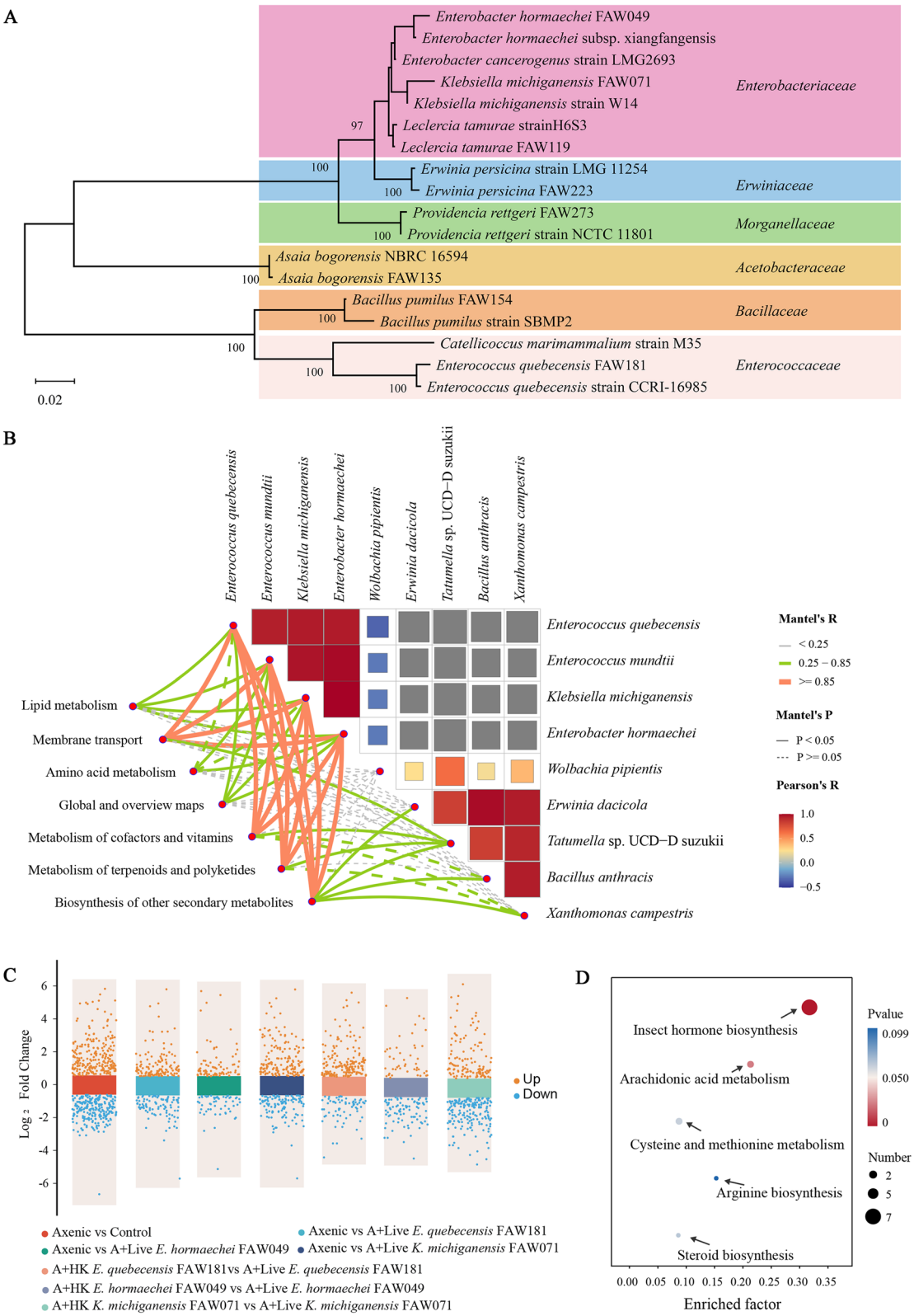

Fig. 5 Effects of gut bacteria on *S. frugiperda* hormone metabolism. **A** Phylogenetic tree containing *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 based on 16S rRNA sequences. **B** Correlation diagram of gut bacterial abundance, with metabolite pathways visualised as network diagrams. **C** Volcano plots of differential haemolymph metabolites across comparison subgroups. **D** KEGG enrichment analysis of differential haemolymph metabolites across comparison subgroups

pathways related to insect hormone biosynthesis and arachidonic acid metabolism (Fig. 5D).

Gut bacteria influence host reproduction by regulating hormones

To determine how hormones affect reproduction in *S. frugiperda*, we analysed metabolites from the JH III and 20E pathways and examined the effects of these hormones through direct injections in adults and the application of their inhibitors (Figs. 6 and S8). Significant differences were observed in JH and 20E pathway metabolites between the haemolymph of axenic and bacteria-containing females. JH III and 20E levels in axenic females and those re-infected with heat-killed bacteria were 5.15–12.65 and 1.79–2.81 times higher, respectively, than those in females with live gut bacteria (control and live re-infection groups; Fig. 6A, B). Conversely, JH III and 20E levels in females re-infected with live bacteria were comparable to those in control females.

Further analysis revealed differential regulation of key precursors in the JH and 20E biosynthetic pathways. Methyl farnesoate, a precursor for JH III synthesis, was differentially down-regulated in control females compared to those re-infected with live *K. michiganensis* FAW071 (Fig. S7A). Similarly, precursors for 20E synthesis, including 2,22-dideoxyecdysone, 3-dehydro-2-deoxyecdysone, and ecdysone, exhibited comparable down-regulation in females with live gut bacteria (control and re-infected with *E. quebecensis* FAW181, *E. hormaechei* FAW049, and *K. michiganensis* FAW071) (Fig. S7B). In contrast, 2-deoxyecdysone displayed variable levels of up-regulation among these groups (Fig. S7B). These data suggest that the gut bacteria *E. quebecensis* FAW181, *E. hormaechei* FAW049, and *K. michiganensis* FAW071 exert direct regulatory effects on JH III levels. Moreover, these bacterial strains modulate the levels of precursors for 20E synthesis, thereby indirectly influencing 20E levels. Collectively, these regulatory mechanisms contribute to the maintenance of JH III and 20E homeostasis in *S. frugiperda*.

Injections of JH III, 20E and JH III + 20E into control adults caused a dose-dependent reduction in *S. frugiperda* fecundity. At JH III, 20E and JH III + 20E injection concentrations ≥ 0.5 μg/μL, egg production per female decreased significantly by 42.80%, 30.62% and 48.06%, respectively (all $P < 0.001$; Fig. S8A). These hormonal treatments also led to a shortened oviposition period and an extended pre-oviposition period (Fig. S8B–C). Interestingly, the injection of these hormones raised JH III and 20E titers in control adults to levels comparable to those observed in axenic adults. Conversely, the application of pharmacological inhibitors (Pre, Aza and Pre + Aza) markedly lowered JH III or 20E titers in axenic adults (Fig. 6C, D). Injections of JH III and JH III + 20E also significantly reduced ovary length, shortened the oviposition period and extended the pre-oviposition period, resulting in a significant reduction in egg production, whereas inhibitor injections partially restored *S. frugiperda* fecundity, although not to control levels (Fig. 6E–H).

These findings suggest that gut bacteria play a critical role in regulating hormonal balance, which is essential for host reproduction. The strains *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 appear to stabilise JH III and 20E titers by modulating the insect hormone pathways, thereby enhancing the fecundity of *S. frugiperda* (Fig. 7).

Discussion

High fecundity is a critical factor in enabling insect populations to adapt to new habitats, supporting rapid population growth and outbreaks, with severe impacts on agroecosystems [52, 53]. Symbiotic gut bacteria play a key role in enhancing host fitness by participating in metabolism, providing essential nutrients and detoxifying secondary metabolites from host plants [54–59]. Advances in next-generation sequencing, including 16S rRNA–based and metagenomic analyses, have become invaluable for understanding the complex interplay between insects and their gut symbionts [60]. Here, we carried out a comprehensive analysis of symbiotic gut bacteria in *S. frugiperda*, revealing their pivotal role in the reproductive processes of their host.

To better understand the interaction between gut bacteria and the host, we examined the dynamics of gut bacterial communities throughout the life cycle of *S. frugiperda*. The bacterial community present in the egg stage is then initially formed and is analogous in structure to that of the lower larval gut flora, suggesting that gut bacteria can be transmitted vertically. The transmission of

(See figure on next page.)

Fig. 6 Effects of JH III and 20E on *S. frugiperda* fecundity. Analysis of JH III (**A**) and 20E (**B**) levels under different treatments. JH III (**C**) and 20E (**D**) titers following injections. Treatments consisted of control (females injected with DMSO), control females injected with JH III, 20E and JH III + 20E, axenic (females injected with DMSO), axenic females injected with Pre (JH biosynthesis inhibitor), Aza (20E biosynthesis inhibitor) and Pre + Aza. Effects of hormone injections on egg production (**E**; $n = 30$), ovarian length (**F**; $n = 20$–24), oviposition period (**G**; $n = 30$) and pre-oviposition period (H; $n = 30$). Data analysed using one-way ANOVA followed by Tukey's multiple comparison test; different letters denote significant differences

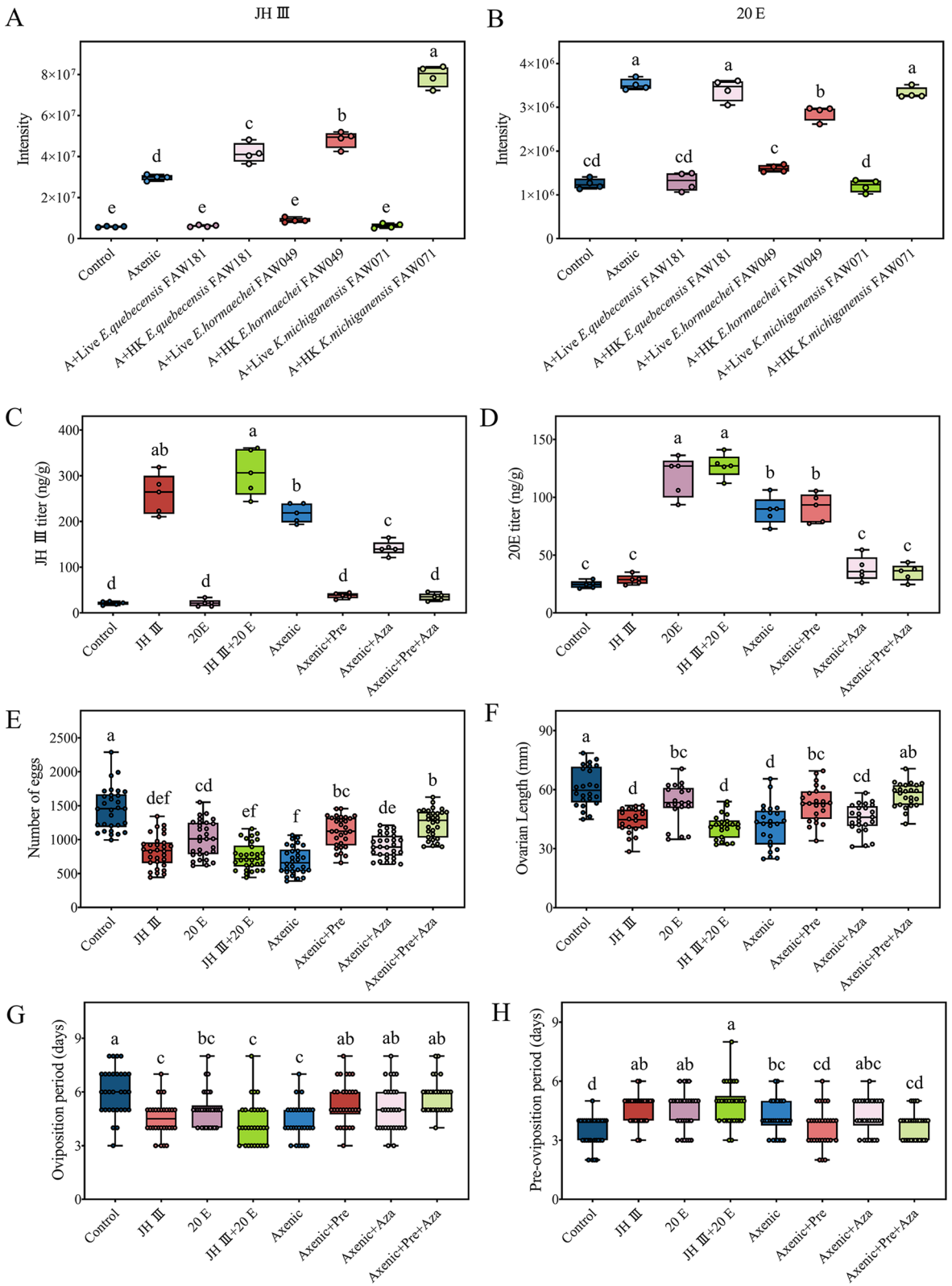

Fig. 6 (See legend on previous page.)

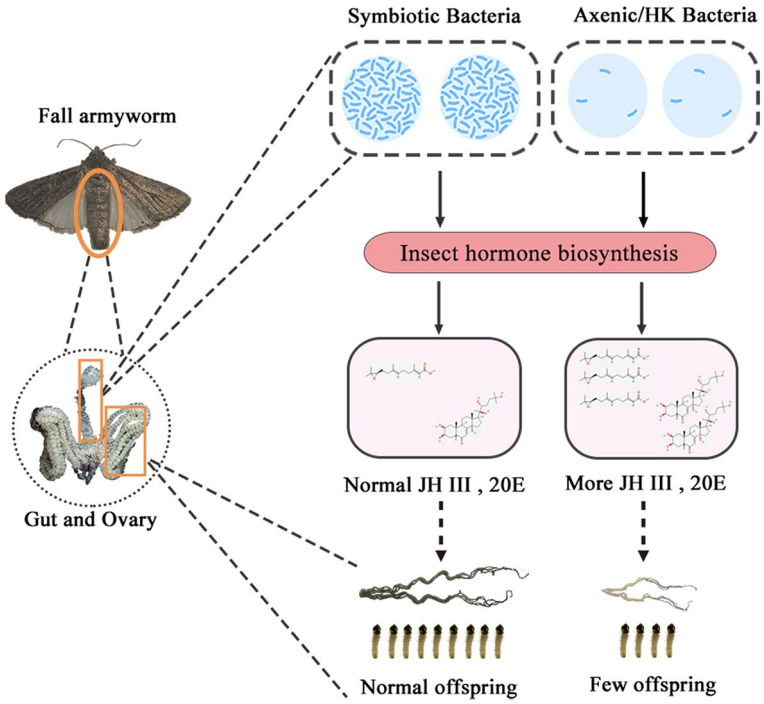

Fig. 7 Schematic overview of how the gut bacteria (*E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071) drive *S. frugiperda* reproduction

Rickettsia occurs in synchrony with the oogenesis of the insect host and the subsequent embryonic development of its progeny [61]. Additionally, *Pantoea dispersa* can establish itself within the testes, thereby facilitating the vertical transmission of this bacterium across successive host generations [62]. During the larval stage, gut bacteria primarily enhance host fitness by facilitating digestion and detoxification. For example, *Enterococcus mundtii* in the gut of *Spodoptera litura* produces antimicrobial compounds that protect against gram-positive pathogens [63]. Similarly, *Enterobacter* species encode genes related to detoxification and degradation of kaempferol, a toxic plant compound, thereby improving host fitness in *P. xylostella* [11, 64]. *Klebsiella* and *Enterococcus* assist in cellulose degradation [65, 66], and *K. michiganensis* BD177 helps *B. dorsalis* adapt to low-temperature stress through arginine and proline metabolic pathways [44]. In *S. frugiperda*, *Enterococcus* and *Enterobacter* in the larval gut enable host growth and development under nutrient-poor dietary conditions [17]. We observed the highest diversity of gut microbial communities during the older larval and pupal stages, whereas young larvae exhibited lower diversity, dominated by the core bacteria *Enterococcus*, *Enterobacter* and *Klebsiella* (Figs. 1 and 2). This concentrated community in early larval stages likely supports nutrient accumulation, enabling rapid development and population establishment. Young larvae feed on tender maize tissues, necessitating a higher abundance of these core bacteria for nutrient acquisition during early developmental stages. The composition of gut microbial communities in herbivorous insects is influenced by several factors, including environment, habitat, diet and age [67–71]. As larvae age and shift their feeding preferences to different maize organs, coupled with the need for increased food intake, the microbial diversity and richness in their guts expand. Thus, *S. frugiperda* appears to require a more diverse microbial community to meet its changing physiological and environmental demands as it matures.

Gut microbes are integral not only during the larval stage but also throughout the adult stage. *Enterococcus*, *Enterobacter* and *Klebsiella* are commonly found in the guts of lepidopterans and other insects [72–77]. The successful establishment of an axenic insect system hinges on three key factors: rearing insects under sterile conditions, using effective sterilization techniques, and providing optimal nutrition [20]. Using antibiotics to obtain axenic insects is a common practice in studies of gut microbe-insect symbiosis [62, 78, 79]. In this study, we successfully eliminated these bacteria from the gut of *S. frugiperda* by sterilizing egg surfaces to remove microbes, rearing larvae on diets providing optimal nutrition in a sterile environment, and administering antibiotics at specific stages (Figs. S4 and S6A). Their removal significantly

impaired host ovary development and egg production, whereas re-infection with *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071, or a mixture of these bacterial strains, restored the reproductive functions to normal levels (Figs. 3 and 4). This confirms the critical role gut bacteria in supporting *S. frugiperda* reproduction. Similarly, antibiotic treatment of *Zeugodacus tau* inhibited ovary development and egg-laying in a previous study [80], and the presence of specific bacteria, such as *Acinetobacter soli*, *Acinetobacter ursingii*, *Moraxella osloensis* and *Empedobacter brevis*, in *Henosepilachna vigintioctopunctata* promoted ovarian and spermatogonial development in another study [81]. These findings align with our observations. The diversity of gut bacteria in *S. frugiperda* increased and then declined across its life cycle (Figs. 1 and 2), suggesting distinct roles for gut bacteria at different stages. This dynamic regulation likely reflects an adaptation to match the host's developmental and reproductive needs.

Symbiotic microorganisms are increasingly recognised as vital components of host endocrine systems [82, 83]. In our study, *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 exhibited a strong correlation with pathways for insect hormone synthesis (Fig. 5). Notably, their elimination and re-infection significantly altered titers of JH III and 20E at the third-day post-eclosion (Fig. 6). In female *S. frugiperda* feeding on more nutritious seeds, the titers of JH III and 20E exhibit a dynamic pattern, peaking at 72 h post-eclosion [84]. These hormones regulate not only insect development and metamorphosis but also reproduction [85, 86]. JH is required for vitellogenin synthesis in the fat body, whereas 20E signalling is essential for ovary development in *Tribolium castaneum* [87]. Similarly, gut symbionts in *Riptortus pedestris*, such as *Burkholderia*, promote JH III skipped bisepoxide synthesis, facilitating hexameric-α and vitellogenin accumulation as well as increased egg production [88–90]. Hormones such as JH and 20E regulate insect development, metamorphosis and reproduction via complex mechanisms [91]. Although ecdysteroids are known to stimulate vitellogenesis and JH to promote vitellogenin uptake by the ovaries [86], elevated levels of these hormones can also negatively affect reproduction. For example, increased 20E titers are known to reduce fecundity in *P. xylostella*, a phenomenon exploited for pest control [92]. Similarly, high JH levels enhance vitellogenin or yolk protein uptake by oocytes, whereas high 20E titers cause resorption of immature eggs, ultimately reducing fertility [93]. In *S. frugiperda*, the application of exogenous JH I and JH II, but not JH III, has been shown to promote ovary development [94]. Our findings underscore the negative impact of elevated JH III and 20E levels on *S. frugiperda* fecundity (Fig. 6). However, the gut bacteria *E. quebecensis* FAW181, *E. hormaechei* FAW049 and *K. michiganensis* FAW071 appear to stabilise JH III and 20E titers, supporting host reproductive success.

While our findings indicate that gut microbes influence host reproduction by regulating hormones, the precise manner in which these effects occur within the brain and the underlying molecular basis remains unclear. Consequently, future research should focus on the host–microbe interactions via the gut–brain axis. Moreover, the populations used in our study do not fully represent field populations. Future research will incorporate field studies to investigate the impact of natural environmental factors on the physiology of *S. frugiperda*.

Conclusions

In summary, this study provides a comprehensive comparison of gut bacterial communities across the *S. frugiperda* host life cycle, elucidating the pivotal roles of key gut bacteria in modulating host reproduction. Specifically, the strains *E. quebecensis* FAW181, *E. hormaechei* FAW049, and *K. michiganensis* FAW071 stabilize JH III and 20E titers by regulating JHAMT and concurrently modulating the levels of 20E and its precursors via PHM, thereby enhancing the fecundity of *S. frugiperda*. Our findings elucidate the intricate mechanisms underlying gut bacteria–host interactions and underscore their significance in the colonization and outbreak dynamics of *S. frugiperda*. This research deepens our understanding of symbiotic relationships in insect systems and offers valuable insights for managing migratory pests.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-025-02121-x.

Supplementary Material: Figure S1. Reproductive parameters of *S. frugiperda* under various mating patterns. Figure S2. Verification of microbial re-infection in axenic adults. Figure S3. Total egg production per female in *S. frugiperda* re-infected with different gut bacteria. Figure S4. Differential abundance of gut bacterial genera in axenic and control *S. frugiperda*. Figure S5. Metagenomic analysis of gut microbiota metabolism in *S. frugiperda*. Figure S6. Heatmap of gut bacterial abundance (A) and the top 30 metabolites contributing to haemolymph separation (B) in *S. frugiperda* females. Figure S7. Differential metabolite analysis of JH III (A) and 20E (B) metabolic pathways in *S. frugiperda* haemolymph. Figure S8. Effects of JH III, 20E and JH III + 20E injections on *S. frugiperda* reproduction. Table S1. Relative abundance of 19 representative bacterial strains isolated from the gut of control *S. frugiperda*. Table S2. Raw data of all metabolite abundance. Table S3. The fold changes of metabolite concentration in the hemolymph across comparison subgroups. Table S4. Differentially expressed metabolites (DEMs) shared in all comparison groups.

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Authors' contributions
B.C. preparation of the manuscript. B.C. and S.G performed the laboratory experiments and analysed the data. W.H., X.S., X.Y., P.X. and X.Z. coordinated the study and validated the experimental results. C.L. and J.M., coordinated the sample collection., K.W. contributed to the conceptual design of the study, and securing funding support. All authors have read and agreed to the published version of the manuscript.

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Data availability
The 16S rRNA gene sequencing datasets are available in the NCBI database (https://www.ncbi.nlm.nih.gov/sra) with BioProject ID PRJNA1192515. Sequencing data of the metagenomes has been deposited in National Genomics Data Center (https://ngdc.cncb.ac.cn/gsa) and available at PRJCA033384.

Declarations

Ethics approval and consent to participate.
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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