

Structure of gut and ovary, with associated microbiota across life stages in the striped stem borer *Chilo suppressalis* (Lepidoptera: Crambidae)

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Abstract

The striped stem borer *Chilo suppressalis* (Lepidoptera: Crambidae) was one of the most serious pests of water-oat *Zizania latifolia*. Microbiota associated with gut, ovary and egg influenced a wide variety of their host traits (such as fitness and immunity), and understand the microbiota structure and its dynamics across *C. suppressalis*'s life was a prerequisite for comprehending the symbiotic relationship between *C. suppressalis* and its microbiota as well as transmission pattern. Herein, we characterized the structure of the gut, ovary and associated microbiota of a striped stem borer *C. suppressalis*. The gut was structurally divided into foregut, midgut and hindgut, and its structure and ultrastructure were very different between adults and larvae, however, this was not documented in detailed before. Microbiota in gut, ovary and egg showed variation in relative abundance. Proteobacteria and Firmicutes became the predominant phyla, and Bacillaceae, Enterobacteriaceae, Enterococcaceae, Halomonadaceae, Moraxellaceae and Streptococcaceae were shared among the gut, ovary and egg of *C. suppressalis*, although they exhibited different relative abundance. The highest bacteria diversity was found in the larval midgut. The bacterial genera distribution showed great differences due to developmental stage, diet and gut compartments. Our results demonstrated that the developmental stage, diet and gut compartment had a considerable impact on gut microbiota of *C. suppressalis*. Genera *Klebsiella*, *Enterococcus*, *Bacillus*, *Citrobacter* and *Lactococcus* were core microbiota, which transferred from the gut to the ovaries and eggs. Our study provided an important insight into investigation of insect-bacteria symbioses as well as its transmission pattern, so as to perform effective biocontrol of this species.

Introduction

The insects' gut is a tube opening from the mouth to the anus, and is divided into foregut, midgut and hindgut. The midgut and hindgut are major regions; useful materials (nutrients) are absorbed in the midgut, and partial nutrients and water are absorbed in the hindgut. Variations in guts among different species can be attributed to differences of feeding biology and food types. The solid-food feeding species usually possess relatively short, thick guts, whereas the fluid feeding species generally have long narrow guts with some modified structures¹.

The gut is a desirable, nutrient-rich ecological niche where multiple microbiota flourish and reproduce, of which the midgut is the main site harbouring various microbiota; the anterior hindgut is the most densely symbiont-inhabited site, due to the available, partially digested food being from the midgut. Conversely, those microbiota may help their hosts in utilizing life-stage-specific resources by providing various functions related to nutrient supplementation^{2,3,4}, detoxification^{5,6}, reproduction⁷ and population differentiation^{8,9}, and this is especially obvious in phytophagous insects. Gut microbes can be transmitted from one generation of the host to the next, a process known as vertical transmission or inheritance^{10,11}. Vertical transmission of microbiota provides a direct way to start a gut microbiome^{12,13}. Yet, the gut microbiota may partly assemble from the food and local environments in each generation¹⁴.

Zizania latifolia (Gramineae: Oryzeae) is an asexual aquatic vegetable with an edible swelling, plump fleshy stem formed by infection of fungus *Ustilago esculenta* to its meristems. The striped stem borer (SSB) *Chilo suppressalis* is one of the destructive generalists of rice *Oryza sativa* in Asia, southern Europe and northern Africa^{15,16,17}. The inter-cropping pattern of rice *O. sativa* and *Z. latifolia* facilitates a transfer of *C. suppressalis* from *O. sativa* plant to *Z. latifolia* plant. In comparison, *C. suppressalis* feeding on water-oat fruit pulps possess higher survival rate, pupal weight and shorter developmental duration than those feeding on *O. sativa* plant^{18,19}, thus leading *C. suppressalis* become a most serious pests of *Z. latifolia*. Larvae of this species bore into sheaths, stems and pulps of *Z. latifolia*, resulting in 'dead hearts', 'dead sheaths' and 'boring pulps'^{20,21}, emphasizing the urgency for developing innovative, effective biological prevention strategies to this pest.

Diet is a major factor in structuring microbiota across animal taxa²², and diet shift results in a microbiota variation across developmental stages of a single host. Larvae of *C. suppressalis* chew the solid parts of plants, while the adults suck nectar or fruit juice. Nutritional or chemical differences of the diets between larvae and adults might significantly effect on their microbiota resided at different stages. Characterization of microbiota structure is a prerequisite for understanding how a microbiota population functions in the gut ecosystem. To date, some documents focused on resistance affect on gut microbiota^{23,24} or diets on larval gut microbiota of *C. suppressalis*^{9,25}. However, structure of the gut and ovary, and comprehensive characterization of gut microbiota dynamics across life history of *C. suppressalis* is not yet available.

In this study, we illustrated the gut and ovary, and systematically profiled the gut microbiota of different life stages (i.e., larvae, adults, ovary and eggs) of *C. suppressalis*, using light and electron microscopes, transmission electron microscope and high throughput pyrosequencing of microbiota 16S rRNA gene fragments. Our data will be informative to better understand the functional role of each organ and associated community shifts during life stage transitions and community structure associated with dietary regimes; thus provide ideas for controlling this pest based on the source and transmission of gut microbiota.

Materials and methods

Plant obtain

Ten clusters of water-oat *Zizania latifolia* variety 'Zhejiao No. 7' were obtained from the field of the Jinhai Yao, a farmer who has long been cultivated *Zizania latifolia* and cooperated with our team in terms of technical guidance of *Z. latifolia*. The obtained 'Zhejiao No. 7' were cultivated in the plant land of our laboratory experimental field for the subsequent investigation. Thus a permission was not required to collect the plants. A good management such as water, fertilizer provision and weed removal was carried out in the field. During the laboratory experiment, pulps of *Z. latifolia* variety "Zhejiao No. 7" were collected from our laboratory experimental field, and the use of the plant parts in the present study complies with laboratory guidelines.

Specimen collection and rearing

Larvae of *C. suppressalis* were collected from the *Z. latifolia* field in Lishui, Zhejiang, where large areas of *Z. latifolia* are exclusively planted.

Larval *C. suppressalis* were reared exclusively with fresh pulps of *Z. latifolia* "Zhejiao No. 7" at $28 \pm 1^\circ\text{C}$, with a photoperiod of 16 h: 8 h (light/dark), and a relative humidity > 80%. All the larvae were reared in the laboratory for three continuous generations and the 4th instar individuals were sampled.

Newly hatched larvae of *C. suppressalis* were introduced into canned bottles (diameter: 10.0 cm, height: 7.0 cm) where fresh pulps of *Z. latifolia* were provided. The bottles were incubated under conditions of $28 \pm 1^\circ\text{C}$, with a photoperiod of 16 h: 8 h (light: dark), and a relative humidity > 85%. Replace water-oats residues by fresh ones every three days. Repeating this procedure until the larvae enter into pupae stage. Picking out the pupae and put them in clean petri dishes (8.8 cm in diameter), containing a small chunk of watery sponge. The petri dishes were kept at the same incubated condition. Female adults were emerged from the pupae after 6 days. Females were fed with 8.5% sucrose water and paired to males for oviposition.

Light microscopy

After three generations of rearing, the females and larvae of *C. suppressalis* were anesthetized for dissection. Under a Motic SMZ168 Stereoscopic Zoom Microscope, the whole guts of both females and larvae and reproductive systems of females were respectively dissected out in a phosphate buffered solution (PBS, 0.2 M, pH 7.2). The guts and reproductive systems were carefully plated into concave dishes separately, and were observed structurally. Related photographs were taken via a Scientific Digital Micrography System (SDMS) which was equipped with an Auto-montage imaging system and a high sensitive Qimaging Retiga 2000R digital camera.

Transmission electron microscopy (TEM)

The guts and ovaries were respectively fixed with 2.5% glutaraldehyde for 12 h at 4°C . Then they were rinsed three times in phosphate buffer (PBS, 0.1 M, pH 7.2), and were post-fixed in 1% osmium tetroxide (PBS, 0.1 M, pH 7.2). After three times' rinse, the guts and ovaries were dehydrated in series of ethanols (30–100%, v/v), and were embedded in Epon 812 for incubation¹. Ultra-thin sections were stained with uranyl acetate and lead citrate, and were observed by a transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan) at 80 kV.

Gut, ovary and egg samples collection

Healthy, uniformly developed eggs, adults and larvae of the same batch of *C. suppressalis* were collected, and were externally sterilized with 75% ethanol and rinsed 3 times with sterilized water. The sterilized eggs were directly placed in sterile microcentrifuge tubes. Individuals of the adults and larvae were anesthetized by placing on ice, and their guts and ovaries were dissected out with a sterilized fine-tip forcep. The guts and ovaries were carefully separated and placed in different sterile microcentrifuge tubes. They were immediately frozen in liquid nitrogen and stored at -80°C for DNA isolation. Six groups of samples (larval midguts, larval hindguts, adult midgut, adult hindguts, ovaries and eggs) were set; the midguts, hindguts and ovaries of 50 individuals, and 100 eggs were respectively collected as one group of sample, and three replicates were taken.

DNA isolation, 16S rDNA amplification

Total DNA was extracted from six groups of samples (guts, ovaries and eggs) using a Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) followed the manufacturer's instructions. Purity and concentration of the DNA were measured by a NanoDrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA)⁹.

Variable V3 – V4 regions of the bacterial 16S rRNA were amplified using two universal primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGTATCTAAT-3'). The Polymerase Chain Reaction (PCR) reaction solution was composed of 4.0 µL 5×FastPfu Buffer, 2.0 µL 2.5 mM dNTPs, 0.4 µL FastPfu Polymerase, 0.8 µL Primer (5.0 µM) and 10 ng template DNA⁹. The amplification procedure was 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. The product was kept at 72°C for 5 min to extent completely⁹.

Illumina MiSeq sequencing

Concentration of the PCR amplifications was examined using 2% agarose gel electrophoresis and quantified via NanoDrop 2000. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq6000 platform according to the instructions.

Processing of sequencing data

Raw sequencing reads were processed in Qiime (v1.9.1) for quality control, filtering, splicing, chimera removal, and sequence optimization.⁹. Reads being not assembled were discarded. Operational Units (OTUs) clustering was performed by UPARSE (v7.1 <http://drive5.com/uparse/>) and chimeric sequences were removed by UCHIME. RDP Classifier (v2.2) Bayesian algorithm and Qiime (v1.9.1) software were used for phylogenetic affiliation of each gene sequence and comparison annotation of species and .

Results

General structure of adult gut

The adult gut was structurally divided into foregut, midgut and hindgut. The foregut contained a narrow oesophagus and a bag-like crop; the oesophagus proceeded posteriorly into the crop and extended into the anterior of the midgut; the pouch-shaped midgut was well-developed, which began from the crop and extended to the hindgut; the hindgut consisted of a long convoluted ileum and a well-developed rectal pouch. Malpighian tubules emerging from the junction of the midgut and the ileum, ran laterally along the midgut in the posterior direction. After reaching the anterior midgut they followed their own course in a posterior direction and gather near the rectum (Fig. 1A).

General structure of larval gut

The gut of larvae was a continuous tube, which ran from the mouth to the anus. Structurally, the gut was divided into three distinct segment: foregut, midgut and hindgut. The foregut was slender and elongate, expanded posteriorly and constricted at its end. The midgut was well-developed and saclike in shape, which began from the end of the foregut and extended to the anterior of the hindgut. The hindgut is about one third the length of midgut, and narrower than the midgut. When the individual was freshly dissected out, the three segments were very different in their color and easy to be differentiated: the foregut was translucent, the midgut was opaque white, and the hindgut was yellowish-brown (Fig. 1B).

General structure of female reproductive system

The female reproductive system was complex for consisting of the following characteristics (Fig. 1C). An ovary (Ov) contained four ovarioles; pedicel of each ovariole connected directly with a lateral oviduct (LO) and finally connected with a common oviduct (CO). Basal region of the common oviduct entered into a genital chamber (GC), which laterally connected with a spermatheca duct (SD). The spermatheca consisted of a principal spermatheca (PSp) and an accessory spermatheca (ASp); a convoluted spermatheca gland (SpG) emerged from the former apex. A duct of accessory gland (DAG) emerged at the lower middle region of the genital chamber, and apically bifurcated to a pair of accessory gland reservoirs (AGR). A coiled accessory gland (AG) situated basally from the reservoir. An afferent duct (AD) emerged from the other sides of the genital chamber, and its distal area connected with a duetus bursae (DB) enlarging distally to a sac-like corpus bursae (CB).

Ultrastructure of the midgut and hindgut of adult *C. suppressalis*

The midgut consisted of two types of cells resting on a basal lamina ($\sim 0.5 \mu\text{m}$). In the first type of cells, round or gourd-shaped nuclei with clumps of heterochromatin bounded by nuclear membrane were found adjacent to the middle region (Fig. 2A). Narrow, long and well-developed basal infoldings ($\sim 2.5 \mu\text{m}$ long) associated with elongated mitochondria are observed at the basal region (Fig. 2A, B). Densely packed microvilli ($\sim 8.5 \mu\text{m}$) were obvious at the apical area (Fig. 2A). The mitochondria occupied the most part, arranging in a row below the microvilli and tending to orientated with their long axes in the direction of the microvilli (Fig. 2B). The cytoplasm contained extensive rough endoplasmic reticulum, electron-dense secretory granules and abundant electron-lucent secretory vesicles (Fig. 2C, D). These secretory vesicles vary in size and shape, and it appears that some small vesicles fused to become larger ones (Fig. 2C, 3B). Many electron-dense lysosome-like granules containing debris of organelles were also observed in the cytoplasm (Figs. 2E, 3A).

In comparison, cells of the second type possessed very shallow basal labriths, though their apical border bears well-developed microvilli (Fig. 3C). Heavy staining of lateral cell membranes were observed in the cytoplasm (Fig. 3D). Desmosomes cannot be discerned due to the over-staining of the membranes by osmium. Large areas of well-developed rough endoplasmic reticulum were found in the cytoplasm (Fig. 4A). Accumulations of glycogen granules ($\sim 50.0 \text{ nm}$ in diameter), electron-lucent secretory vesicles were present in the cytoplasm (Fig. 4A). These particles were spherical or possibly polyhedral. Electron-dense secretory granules, mitochondria with cristae scattered through the cytoplasm (Fig. 4B). Small secretory granules seemed to fuse into bigger ones. Nuclei with small nucleoli were bounded by nuclear membrane (Fig. 4B, C). The cytoplasm seemed to be ruptured due to the load of virus-like particles. Electron-dense secretory granules, mitochondria with cristae, and nuclei with small nucleoli were bounded by nuclear membrane (Fig. 4B, C). Microorganisms ($\sim 2.4 \mu\text{m}$ long, $0.2 \sim 0.5 \mu\text{m}$ in diameter) scattered among the mitochondria (Fig. 4A, D).

The ileum was lined with a highly convoluted cuticle containing an electron-dense epicuticle ($0.5 \mu\text{m}$) and an electron-lucent endocuticle ($\sim 0.3 \mu\text{m}$) facing the lumen (Fig. 5A). Cells beneath the cuticle were featured by elaborated extensive apical leaflets formed by invagination of the apical plasma membrane. Abundant mitochondria packed the cytoplasm and were associated with the leaflets. The basal plasma membrane invaginated into a few extremely wide and very shallow infoldings associated with mitochondria. Large numbers of oval or spherical microorganisms with electron-dense membrane were observed in the lumen of this organ (Fig. 5B). Within the membrane, electron-lucent vesicles and brush-like structures adjacent to the particles were also present (Fig. 5B).

Ultrastructure of the midgut and hindgut of larvae *C. suppressalis*

Cell of the midgut rested on a basal lamina ($\sim 0.2 \mu\text{m}$), and its basal region was equipped with elongated mitochondria with evident cristae (Fig. 6A). Among the mitochondria, rough endoplasmic reticulum was scattered. Apical plasma membrane beared well-developed apical microvilli, (about $2.5 \sim 4.0 \mu\text{m}$ long), adjacent to which were some oval mitochondria (Fig. 6B). The mitochondria and rough endoplasmic reticulum occupied the most part of the cytoplasm.

Cells of the hindgut rested on a layer of very thin basal lamina ($\sim 0.05 \mu\text{m}$) (Fig. 6C). Basal plasma membrane invaginated into very wide infoldings associated with oval mitochondria and rough endoplasmic reticulum; whereas the apical plasma membrane was elaborated to well-developed apical leaflets (Fig. 6D).

Ultrastructure of ovary of female *C. suppressalis*

The ovary contained numerous nurse cells; cylindrical follicle cells with evident nuclei enveloped entirely the oocyte (Fig. 7A, B). Basal membrane of the follicle cell invaginated into long wide infoldings associated with elongated or oval mitochondria (Fig. 7C). Well-developed microvilli were visible at the apical area of the follicle cells, of which the cytoplasm was filled with yolk granules and lipid droplets (Fig. 7A, D, 8A). These granules and droplets vary in size and electron-density, and some smaller ones fused into be larger ones (Fig. 8A). Rough endoplasmic reticulum and virus-like particles scattered among the granules and droplets (Fig. 8B). Abundant elongated or oval mitochondria of various size were visible throughout the cytoplasm. Sparse less-developed apical microvilli and numerous elongate mitochondria seemed to been taking part in the secretion (Fig. 8C). Different from the oocyte, the nurse cell was filled with secretory granules of different size and electron-density (Fig. 8D).

Analysis of bacterial 16S rDNA gene sequences

A total of 687,799 sequences clustering into 2524 operational taxonomic units (OTUs) were generated to analyze to microbiota (Table 1). Detected OTU numbers, Chao and Shannon were calculated as alpha diversity indicators. The relative bacterial abundance of 17 phyla differs significantly among the guts, egg and ovary (*Kruskal-Wallis* test, $p < 0.0001$) (Table S1). Although the bacteria identified in the larval gut (JMG) were more diverse than those in the adult egg (JEG), both organs possessed the highest bacteria diversity and richness (Table 1).

Table 1
Diversity of gut bacterial communities based on sequencing

Developmental stages	Diet type	Sample	Abbreviations	Reads	Bases (bp)	OTUs	Coverage	Richness estimate		Diversity index
								Chao1	S	Shannon
larvae/adults	Pulps	Midgut	JMG1	40138	17434180	248	0.998729	303	40769	1.82
			JMG2	30164	13022139	156	0.998110	225	30361	1.76
			JMG3	38225	16566823	185	0.998483	242	38626	2.03
		Hindgut	JHG1	39198	17058995	154	0.998954	182	39778	1.78
			JHG2	29999	13129341	97	0.999033	142	30626	1.84
			JHG3	41964	18898628	105	0.999261	129	44068	1.45
	No	Midgut	AMG1	29622	13457815	116	0.999122	134	31413	1.29
			AMG2							
			AMG3	31377	14267149	119	0.999076	140	33296	1.31
		Hindgut	AHG1	42226	18171436	94	0.999124	145	42360	1.65
			AHG2	40005	17192557	137	0.998700	178	40081	0.78
			AHG3	36054	15537581	100	0.998835	151	36219	1.35
Ovary/Egg	Ovary	JOV1	38043	16419021	60	0.999448	95	38220	0.86	
		JOV2	42406	18247431	77	0.999245	148	42501	1.00	
		JOV3	37306	16100611	66	0.999383	94	37483	0.89	
	Egg	JEG1	30535	14170587	102	0.999247	115	33125	1.85	
		JEG2	41371	18295563	106	0.999202	150	42683	1.68	
		JEG3	35379	16197473	117	0.998954	159	37890	1.94	
Note: S, Number of Sequences; WO, water-oat; RS, rice seedlings.										

Diversity of gut microbiota

A total of 46 OTUs were shared by all of the samples (gut, ovary and egg) (Fig. 9), and the core OTUs belonged to the four phyla Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (S1 Fig). The OTUs detected from different samples grouped into distinct families with vary abundance: these coming from the midgut contained 85 families, and Enterobacteriaceae (35.93%~94.12%) and Enterococcaceae (3.86%~12.41%) were the most abundant families (Table S2A; Table S3); these from the hindgut were classified into 71 families, with Enterobacteriaceae (67.25%~77.89) and Enterococcaceae (10.4%~11.4%) being predominant families (Table S2B; Table S3); those identified from the egg were classified to 101 families, and the Bacillaceae (32.5%), Streptococcaceae (16.2%), Enterococcaceae (8.3%), Enterobacteriaceae (5.3%) and Flavobacteriaceae (5.1%) become prevalent families (Table S3); these from the ovary were grouped into 96 families, with Enterobacteriaceae (40.7%), Enterococcaceae (28.8%) and Bacillaceae (13.1%) become the predominant families (Table S3).

OTUs from the different gut compartments exhibited an great different within larvae or adult: these from the adult hindgut (AHG) were grouped into 45 families, and Enterobacteriaceae (78.4%), Enterococcaceae (11.5%) and Bacillaceae (4.0%) were the predominant ones (Table S3); those from the adult midgut (AMG) were classified as 35 families, of which Enterobacteriaceae (94.1%) was the dominance taxa (Table S3). In comparison, OTUs obtained from AHG were grouped into 67 families, with Enterobacteriaceae (66.7%), Bacillaceae (11.3%), Enterococcaceae (10.1%) and Streptococcaceae (5.9%) being prevalent families (Table S3); these from the larval midgut (JMG) were classified into 84 families, and Enterobacteriaceae (35.9%), Bacillaceae (26.9%), Streptococcaceae (14.1%), Enterococcaceae (12.4%), and Halomonadaceae (4.9%) become the predominant families (Table S3).

Taxonomic distribution of gut microbiota of *C. suppressalis*

In terms of phylum composition, a homogeneous distribution was found in all samples (AHG, AMG, JMG, JEG, JOV) and larval hindgut (JHG): Proteobacteria (8.6–96.3%), Firmicutes (2.5–89.7%), respectively (Fig. S1; Table S4); and the two predominant phyla 'Proteobacteria and Firmicutes' changed significantly between the JEG and JMG (Figs. 10, S1; Table S3 and Table S4). A total of 17 bacterial phyla grouped into 129 families, and the most predominant family was Enterobacteriaceae, followed by Bacillaceae and Enterococcaceae (Table S3). The families Bacillaceae, Enterobacteriaceae, Enterococcaceae, Halomonadaceae, Moraxellaceae, Streptococcaceae and Pseudomonadaceae were found across all samples (AHG, AMG, JHG, JMG, JEG, JOV), although they exhibited different relative abundance; but the Brucellaceae, Clostridiaceae, Lactobacillaceae, Microbacteriaceae and Planococcaceae were only found in the JEG, JOV, JHG, JMG, although it was low in relative abundance (Figs. 10, 11; Table S3). Bacillaceae was enriched in the egg (32.5%), followed by the JMG (26.9%) and JOV (13.1%). Enterobacteriaceae was enriched in the AMG and AHG (94.1%, 78.4%) and JHG (66.7%), respectively. Enterococcaceae was predominant in the JEG (28.8%), JMG (12.41%) and AHG (11.5%). Halomonadaceae and Streptococcaceae were abundant in the JOV (5.8%, 6.4%), JEG (5.2%, 16.2%) and JMG (4.9%, 14.1%), respectively. Moraxellaceae was enriched in the JEG (4.4%), and presented with a lower abundance in JOV, AHG and JMG (0.4%, 0.2%, and 0.2%). Some families have a more lower abundance, for example, Brucellaceae in the JEG (0.4%) and JOV (0.2%), Clostridiaceae, Lactobacillaceae in the JMG (0.2%, 0.7%) and JEG (0.2%, 1.0%), Microbacteriaceae and Planococcaceae in the JEG (0.2%), and the Pseudomonadaceae in the JOV (0.4%) and JMG (0.4%). These bacteria exhibited a variation of relative abundance associated with developmental stage, diet and gut compartment.

Based on analysis of developmental stage and gut compartments, the bacterial genera from adults and larvae showed distinct distribution (Fig. 11; Table S5). *Klebsiella* was dominant in the AMG (64.3%) and AHG (75.6%), followed by *Enterococcus* in the AHG (11.4%) and *Morganella* in the AMG (12.4%). *Citrobacter* and *Enterococcus* in the AHG (5.1%, 11.4%) has a higher abundance than those in AMG (2.2%, 3.8%). In the JEG, *Bacillus* (32.5%) was prevailed, followed by *Lactococcus* (15.8%), *Enterococcus* (8.3%) and *Halomonas* (5.2%). *Citrobacter* was dominant in the JHG (40.81%), followed by *Klebsiella* (25.7%). In the midgut of larvae, *Klebsiella* (35.1%) was dominant in the JMG, followed by *Bacillus*, *Lactococcus* and *Enterococcus* (26.9%, 13.9%, 12.4%). *Halomonas* was present in the JHG and JMG with a lower abundance (3.4%, 4.9%). *Enterococcus* was notably dominant in the JOV (28.4%), followed by *Klebsiella* (28.4%), *Bacillus* (13.1%) and *Lactococcus* (6.3%); *Halomona* and *Morganella* were presented with a similar predominance (5.8%). *Providencia* was only identified in the AMG, AHG and JOV (3.7%, 5.6%, 8.2%), respectively.

Development-, compartment- and diet-related variations in the microbiota

In *C. Suppressalis*, 99 bacterial genera were identified, and influence of compartment proved to be significant with a well-defined cluster formed within larvae and adults (i.e., JHG, JMG or AHG, AMG). Bacteria from the JOV and JEG were more heterogeneous for constituting clusters (Fig. 12). Enterobacteriaceae, Bacillaceae and Streptococcaceae exhibited a significant difference in abundance among the guts. Enterobacteriaceae was dominant in the AHG and AMG (78.4%, 94.1%), but it was decreased in the JHG, JMG (66.7%, 35.9%). In comparison, Bacillaceae was less abundant in the AHG and AMG (4.0%, 0.7%), whereas it was increased

significantly in the two regions of the larvae (11.3%, 26.9%). Streptococcaceae and Halomonadaceae were less rich in the AHG (1.9%, 1.6%) and AMG (0.3%, 0.3%), but were increased in the JHG (5.9%, 3.4%) and JMG (14.1%, 4.9%) (Fig. 12; Table S3).

The non-metric multidimensional scaling (NMDS) analysis revealed a clear separation of all samples in accordance to developmental stage and gut regions, and a closer association between JMG and JOV. Clusters were well-defined and the relatively great variable in the samples (i.e., JMG, JHG, AMG, AHG, EGG) excepting for JOV. The JEG cluster exhibited the most different microbiota, followed by the AHG and AMG clusters; the JHG cluster showed an intermediate composition. The JEG and JOV clusters exhibited a higher inter-sample variation; the JHG cluster showed an intermediate composition respecting to the AMG, AHG and JHG clusters. The JMG and JOV clusters were similarly homogeneous; the JEG was the most heterogeneous, followed by JOV (Fig. 13).

Discussion

Morphology and ultrastructure of gut in *C. suppressalis*

In the present study, three types of basal infoldings were observed in the gut cells of *C. suppressalis*: 1) very narrow, long infoldings; 2) shallow, scattered infoldings; 3) extremely wide, very shallow infoldings. The first type of infolding were frequently reported in many insects, such as the filter chamber of leafhopper *Cicadella viridis* and midgut of midge *Belgica antarctica*^{1,26}. The second type of infolding were morphologically similar to those previously found in the midgut of leafworm *Alabama argillacea*²⁷, ileum of *C. viridis*¹ and rectum of hangingfly *Bittacus cirratus*²⁸. Well-developed apical microvilli only observing in the midgut of *C. suppressalis* were similar to those reported in the midgut of katydids *Gampsocleis gratiosa*²⁹ and black soldier fly *Hermetia illucens*³⁰. The microvilli and infoldings can enormously increased the surface area of the cell membrane and indicated a secretory function³¹. However, some investigators suggested that infoldings and microvilli provided “effective coupling of passive solvent movements to active salt transport during fluid”^{32,33}. In our opinion, well-developed infoldings and microvilli, abundant mitochondria, extensive rough endoplasmic reticulum and many secretory vesicles existed in the cytoplasm were probably play a important role during ion transport of midgut cells in *C. suppressalis*.

The most striking character of ileum cells in *C. suppressalis* was well-developed apical leaflets associated with abundant mitochondria, resembling those found in the *C. viridis* and fruit fly *Bactrocera dorsalis*^{1,34}. Such leaflets could greatly increase the contact surface with the luminal fluid³⁵, and the “apical infoldings and their associated mitochondria formed mitochondrial pumps for ion transport from the gut lumen to the hemocoel”³⁶. Thus the extensive apical leaflets in the hindgut cells of *C. suppressalis* might be play a vital role in efficient re-absorption of ions.

Lysosome-like granules were observed in the midgut epithelium of *C. suppressalis*, resemble those in the midgut of cockroach *Periplaneta americana*³⁷ and *G. gratiosa*²⁹. Such structure were probably involved in the ultrastructural changes in aging cells via the activity of phagocytes or autophagy of cells. The presence of such structures suggested the midgut cells of *C. suppressalis* were probably in an aging stage and their ultrastructure is undergoing an alteration.

Morphology and ultrastructure of the ovary of *C. suppressalis*

The female reproductive system of *C. suppressalis* was similar to other lepidopterans, such as *Psilogramma menephron*, *Tirathaba rufivena*, *Holcocerus hippophaecolus*, *Opisina arenosella*^{38,39,40,41}, and they shared the following characters: 1) it consisted of paired ovaries, and each ovary contained three to five ovarioles; 2) two lateral oviducts fused into a common oviduct; 3) a genital chamber connected laterally with a long convoluted spermatheca gland; 4) a spermatheca was composed of a primary gland and an accessory gland. However, there were some differences among the species, such as moths *Grapholita molesta*⁴², *Phauda flammans*⁴³, genus *Palumbina*⁷, *Sphecodoptera shen*⁴⁴ and butterfly *Tirumala limniace*⁴⁵. These differences were probably attributed to the species difference, but needs to be confirmed in the future.

Follicle cells played an important in the formation of egg⁴⁶, and margins of the follicular cells were blurred at the beginning. The distribution of abundant mitochondria and rough endoplasmic reticulum was very similar to that observed in the bug *Lygus lineolaris*⁴⁷, probably suggesting that the follicle cells of *C. suppressalis* need more substances coming from the hemolymph to synthesize precursors of egg, in the early stage of egg shell formation, follicular cells began to secrete and form proteinaceous yolk membrane, as indicated previously by Simiczjew (2003)⁴⁶. With the gradual densification of the yolk membrane, follicular cells might

secrete a dense inner egg shell. Subsequently, the secretions continued to accumulate on the inner egg shell to form the outer egg shell.

Microbiota associated with gut, ovary and egg

In the present study, we characterized the microbiota composition and relative abundance of *C. suppressalis* across its life stage. The phyla Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria together constituted the most microbiota (90.7 ~ 99.9%), suggesting the microbiota was rather conserved in adults regardless of diet shift occurring after the metamorphosis. Bacterial diversity was notably greater in the larval midgut of *C. suppressalis*, comparing with other region or egg. Since midgut was the main region of food digestion, and its pH, physiological habitat and available food facilitated the residence of abundant microbiota. The recurrent detection of *Klebsiella*, *Enterococcus*, *Bacillus*, *Lactococcus* and *Citrobacter* in ovary, egg and gut suggested that they were core microbiota, and may be influenced by compartment, diet, and developmental stage. Since die of the adult and larvae of *C. suppressalis* were very different in nutritional ingredient, and the pulps ingested by larvae contain some secondary compounds (i.e., oxalic acid and tannin); it was probable that the core bacteria identified in *C. suppressalis* gut was potential symbiont or beneficial bacteria, which might have evolved in closely related to hosts or and was simply transient in the gut from the food, and be able to colonize in different gut regions, as indicated by Negash et al. (2020) and Berg (1996)^{48, 49}, further research is necessary to elucidate their potential functions.

The composition and richness of bacterial genera in *C. suppressalis* exhibited significant differences across different life stages (adult, larvae, egg). *Klebsiella* dominated in the AMG, AHG and JMG, were suggested to contribute to nitrogen requirements^{26,50}. *Citrobacter* and *Bacillus* were only prevailed in the JHG and JEG, respectively; *Providencia* was found exclusively in the AMG, AHG and JOV, and their functions merits further investigation. *Enterococcus* was indicated to be associated with insecticide and pathogen resistances^{23,51}, thus the distribution of this genus in all the organ of *C. suppressalis* may closely related to the immune of this pest. Diet and developmental stage modulated microbiota^{52,53,54,55}, and some bacteria probably suppress other phyla in the same habitat⁵⁶. Therefore, we inferred that the different bacteria dominance of *C. suppressalis* might be related to successful reproduction of some bacteria genus and suppression of others species in turn.

Our findings showed that a great variation in different microbiota in the AMG, JMG and JEG. The divergence in the microbiota may reflect divergent functional roles in specific resource use. Diet and developmental stage presumably drives the remarkable difference in microbiota between pulp-feeding larvae and sucrose water-feeding adults of *C. suppressalis*. The difference in microbiome between the AMG and JMG was attributed to be a combination of metamorphosis and diet consumed, as holometabolous insects typically voided their gut contents and lost their gut microbiome during pupation, and the diet is different between larvae and adult. After emergence, adult feeding might stimulate the growth of persisted bacteria, or new bacterial taxa sourced from the diet restoring community richness. The well-defined clusters of gut regions of *C. suppressalis* suggested a stable microbiota profiles, and the bacteria variation could be attributed to acquire microorganisms from a greatly diverse environmental reservoir microflora randomly, as was indicated by Curtis and Sloan (2004)⁵⁷.

The present results illuminated the abundance of two dominant phyla (i.e., Proteobacteria and Firmicutes) and the difference of three families (Enterobacteriaceae, Bacillaceae and Enterococcaceae) in *C. suppressalis*. Proteobacteria was reported to be involved in nitrogen⁵⁸ and carbohydrate degradation, such as starches and hemicellulose⁵⁹. Firmicutes was suggested to take part in energy absorption from the diet and may influence the development⁶⁰. Enterobacteriaceae was known to be involved in pheromone production⁶¹. Our findings suggested that the rapid fluctuation of microbiota from larval to adult guts was probably influenced by the diet shift and microbiota recombination which were important factors in modulating the microbiota, as was documented for other Lepidopterans, Coleopterans and Isopterans^{56,62,63,64,65,53}. The persistent of some bacteria of *C. suppressalis* throughout their life history suggested that these core bacteria could be transmitted to ovaries and eggs, and the bacteria carried by eggs can further spread to the next generation, and may be associated with host adaptation. However, further investigations are needed to examine their stability inside the eggs and in the gut of *C. suppressalis* after multiple generations.

Conclusion

- Structure of gut and ovary, with associated bacteria across life stages in the striped stem borer *Chilo suppressalis* (Lepidoptera: Crambidae)

We investigated the structure of gut and ovary and associated microbiota across life stages in *C. suppressalis*. The comparison of gut structure and ovary with associated microbiota supplied important information about gut structure and microbiota changes due to development-, compartment- and diet-alter of *C. suppressalis*. Analysis of microbiota provided an some vital important about the mechanisms underlying *C. suppressalis* adaptation to development and host plants at the bacterial level. The present study showed that the larval midgut had the highest bacteria diversity; the most prevalent phyla were Proteobacteria and Firmicutes; and the dominant families were Enterobacteriaceae, followed by Bacillaceae and Enterococcaceae. The diet, development and compartment were probably contributed the highly diversified microbiota at the genera level adult and larvae. The bacteria change of microbiota parallels to those of own morphology. These findings not only provided an important insight into investigation of insect-bacteria symbioses but also supplied an effective control of lepidopterans from the aspect of microbiota.

Abbreviations

AHG: Hindgut of adult; AMG: Midgut of adult; JEG: Egg deposited by female; JHG: Hindgut of larvae; JMG: Midgut of larvae; JOV: Ovary of female; NCBI: National Center for Biotechnology Information; NMDS: Non-metric multidimensional scaling; OTUs: Operational Units; PCR: Polymerase chain reaction; SSB: Striped stem borer; SRA: Sequence Read Archive.

Declarations

Competing interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

H.Z. conducted experiments, analyzed data and wrote original draft. J.Z. conceived and designed the research. F.L. sampled the insect and conducted part of the experiments. K.Y. reared and sampled insect, and analyzed data. J.C. reviewed and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Data Availability

The raw reads were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the BioProject: PRJNA1142714. Because the Release date set is 2026-12-31, the deposited data is not accessible until 2026-12-31. <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1142714?archive=sra>

Supplementary information:

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Figures

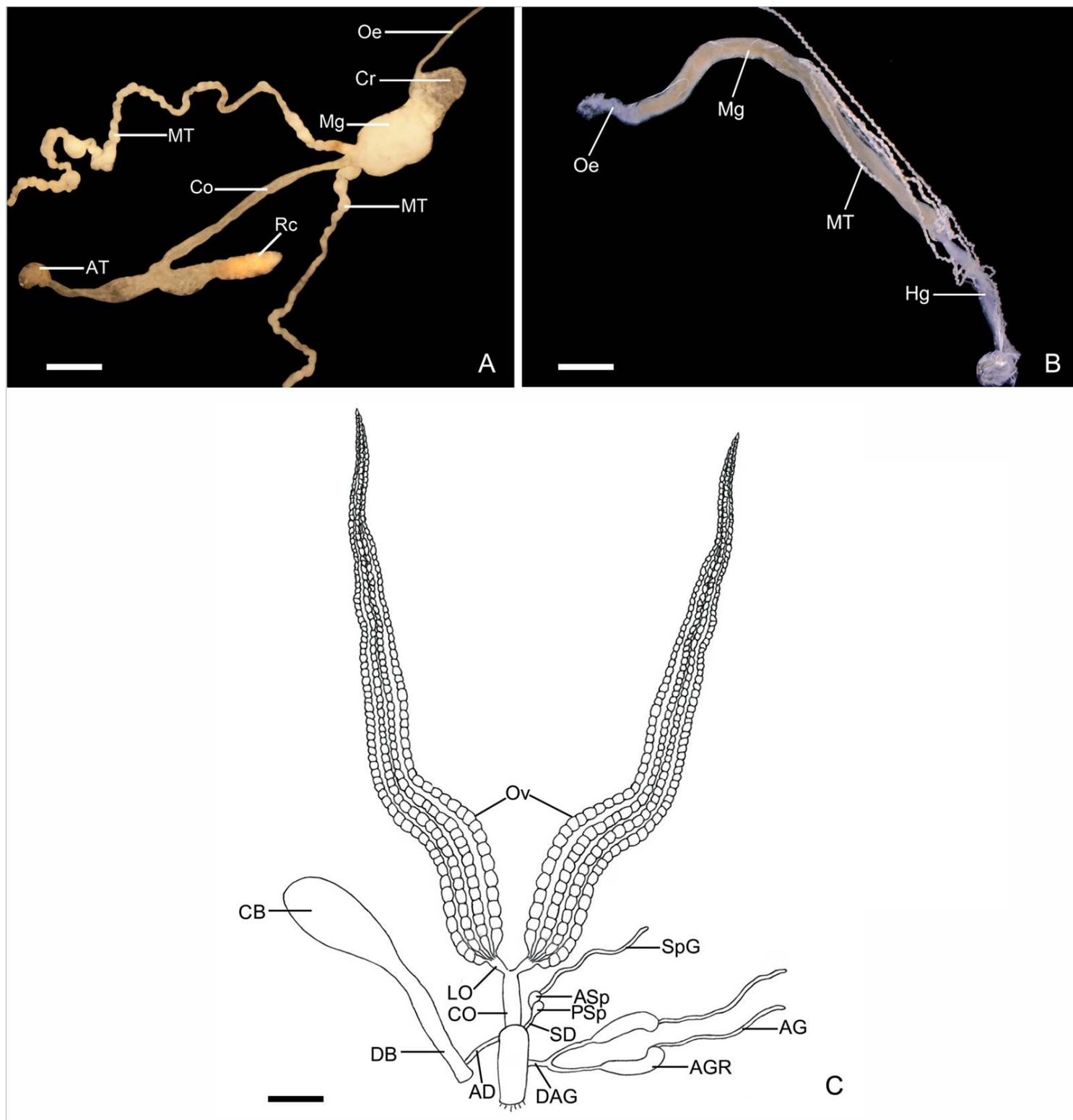


Figure 1

General structure of the alimentary canal and reproductive system of the striped stem borer *Chilo suppressalis*. (A) Adult. (B) Larvae. (C) Reproductive system of female. AD, afferent duct; AG, accessory gland; AGR, accessory gland reservoir; ASp, accessory spermatheca; AT, anal tube; CB, corpus bursae; Co, ileum; CO, common oviduct; Cr, crop; DAG, duct of accessory gland; DB, duetus bursae; GC, genital chamber; LO, lateral oviduct; Mg, midgut; MT, Malpighian tubule; Oe, oesophagus; Ov, ovary; PSp, principal spermatheca; Rc, rectum; SD, spermatheca duct; SpG, spermatheca gland. Scale bars: A–B: 0.4 mm; C: 0.5 mm.

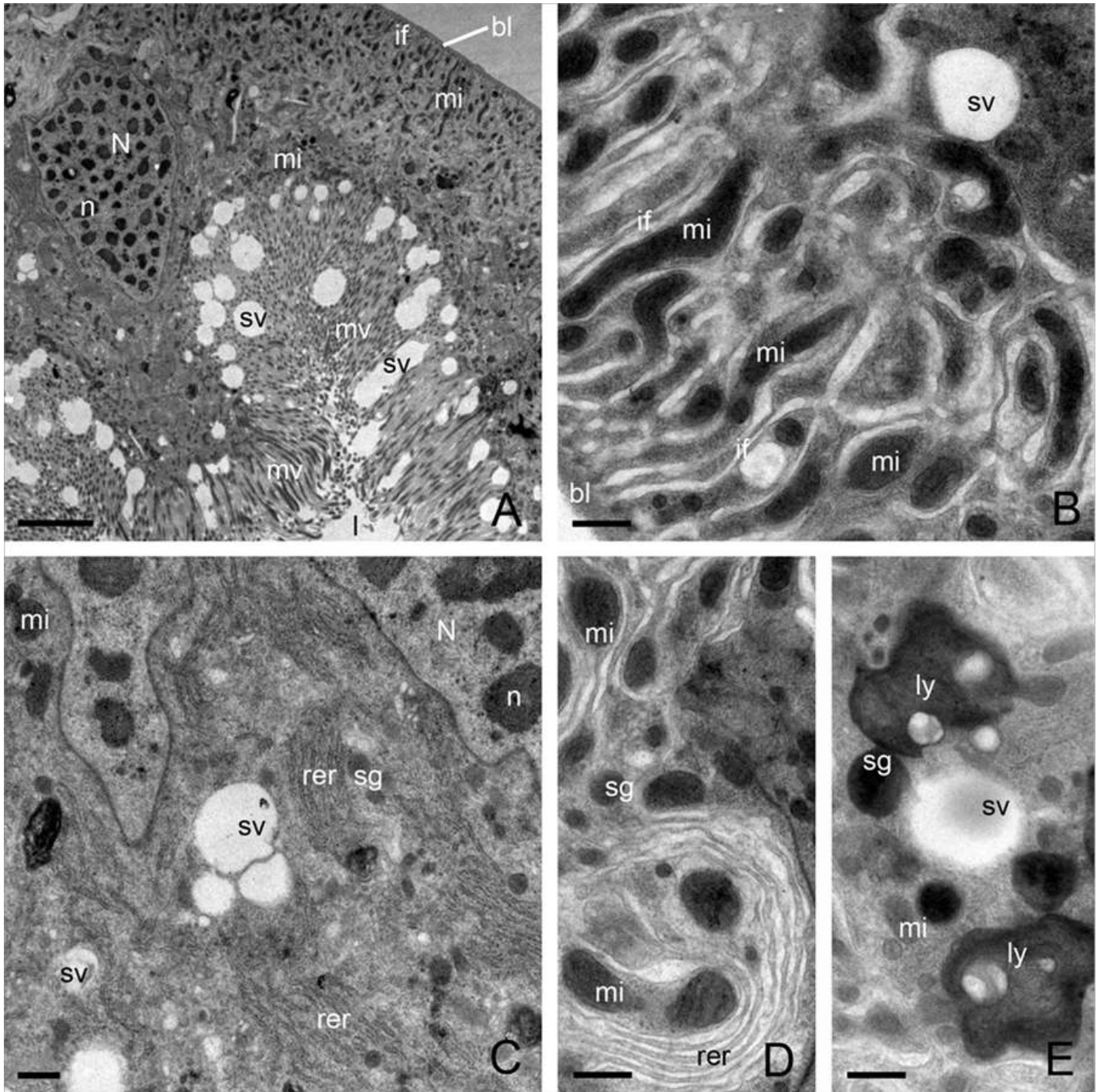


Figure 2

Electron micrographs of the first midgut cells in adult *C. suppressalis*. (A) Transverse section of the midgut. (B) High magnification of the basal region of midgut cells, showing well-developed basal infoldings contain abundant mitochondria and electron-lucent secretory granules. (C-E) High magnification of the middle region of midgut cells, showing scattered nuclei and rough endoplasmic reticulum, secretory vesicles and mitochondria scatter in the cytoplasm. bl, basal lamina; if, basal infoldings; l, lumen; ly, lysosome-like structures; Mi, mitochondria; n, nucleoli; N, nuclei; rer, endoplasmic reticulum; sg, secretory granules; sv, secretory vesicles. Scale bars: A: 5.0 μm ; B-E: 0.5 μm .

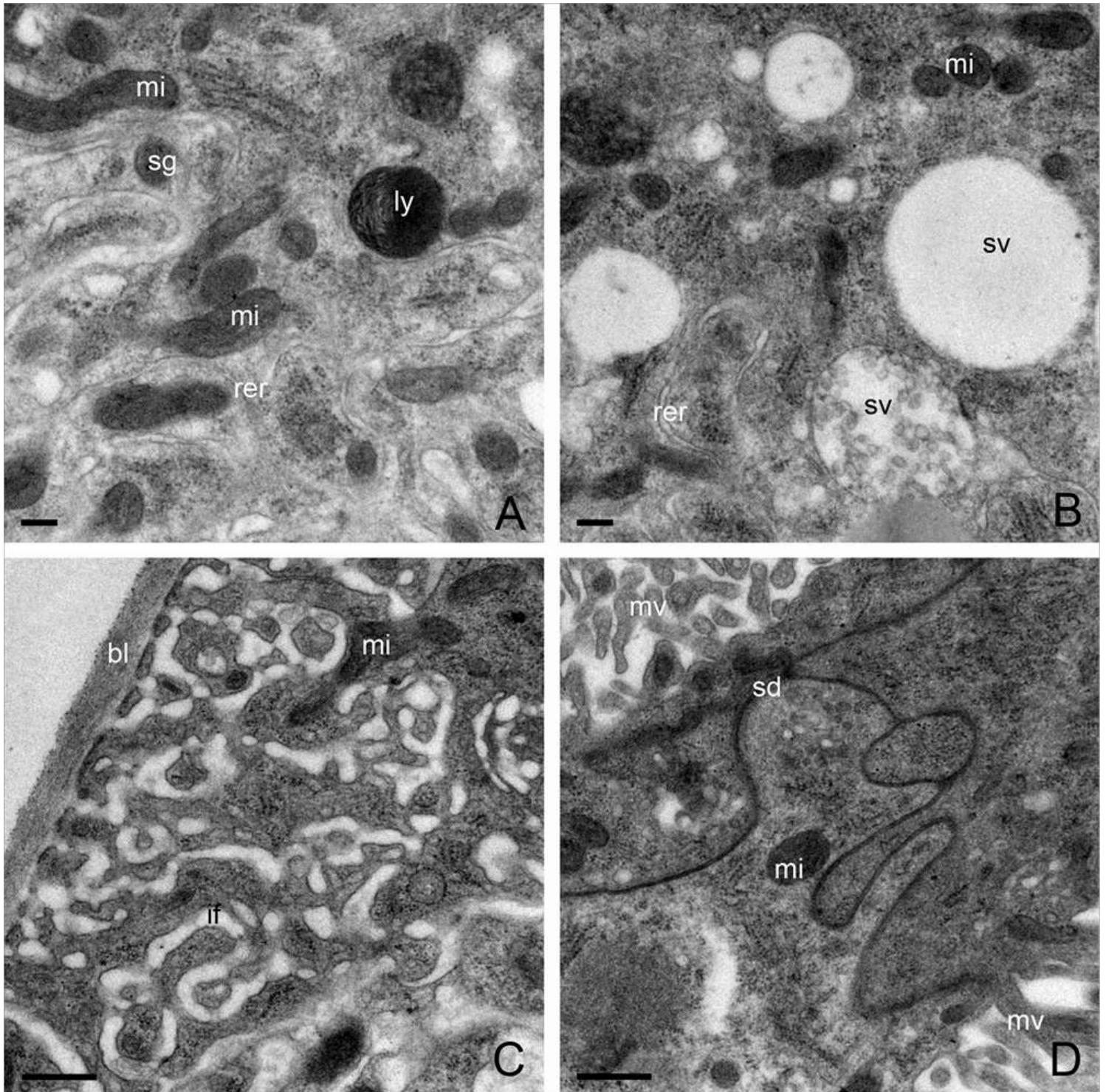


Figure 3

Electron micrographs of the midgut in *C. suppressalis*. (A-B) High magnification of the middle region of the first midgut cell. (C-D) High magnification of the middle region of the second type of midgut cell, showing scattered nuclei and rough endoplasmic reticulum, secretory vesicles and mitochondria scatter in the cytoplasm. bl, basal lamina; if, basal infoldings; lysosome-like structures; Mi, mitochondria; rer, endoplasmic reticulum; sd, septate desmosomes; sg, secretory granules; sv, secretory vesicles. Scale bars: A, B: 0.2 μ m; C, D: 0.5 μ m.

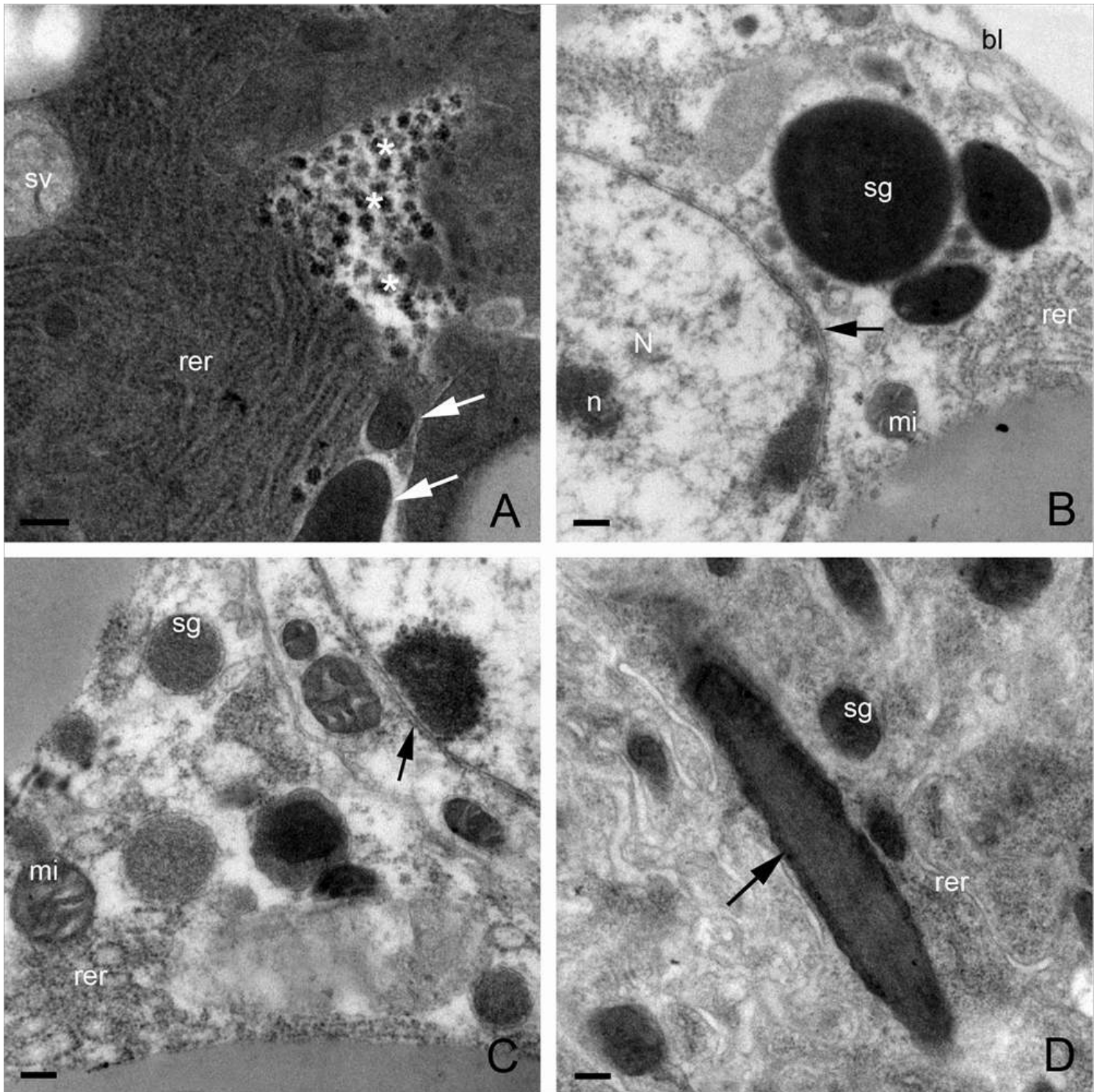


Figure 4

Electron micrographs of the second midgut cells in *C. suppressalis*. (A) Middle region of the first type of cell, showing well developed rough endoplasmic reticulum, accumulations of glycogen granules (asterisks), and electron-lucent secretory vesicles. Arrows indicate microorganisms. (B-C) Close-up of the nucleus, secretory granules and mitochondria. (D) Close-up of a microorganism. bl, basal lamina; Mi, mitochondria; n, nucleoli; N, nuclei; rer, rough endoplasmic reticulum; sg, secretory granules; sv, secretory vesicles. Nuclei are bounded by nuclear membrane (as arrows indicates). Scale bars: A-D: 0.2 μ m.

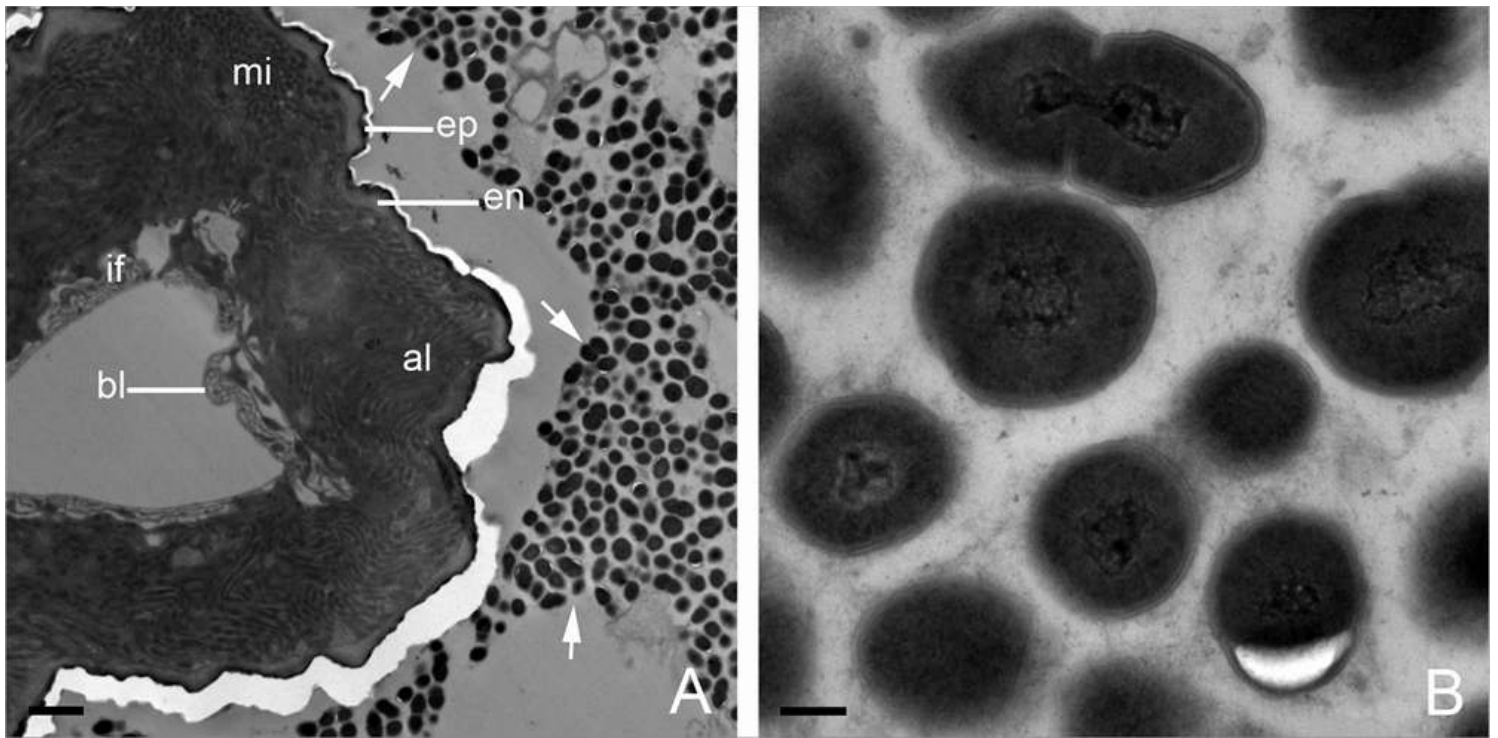


Figure 5

Electron micrographs of the hindgut in *C. suppressalis*. (A) The ileum cells contain abundant mitochondria. al, apical leaflets; bl, basal lamina; en, endocuticle; ep, epicuticle; if, basal infoldings; Mi, mitochondria. Arrows indicate microorganisms in the lumen. (B) Close-up of microorganisms in the lumen. Scale bars: A: 2.0 μm ; B: 0.2 μm .

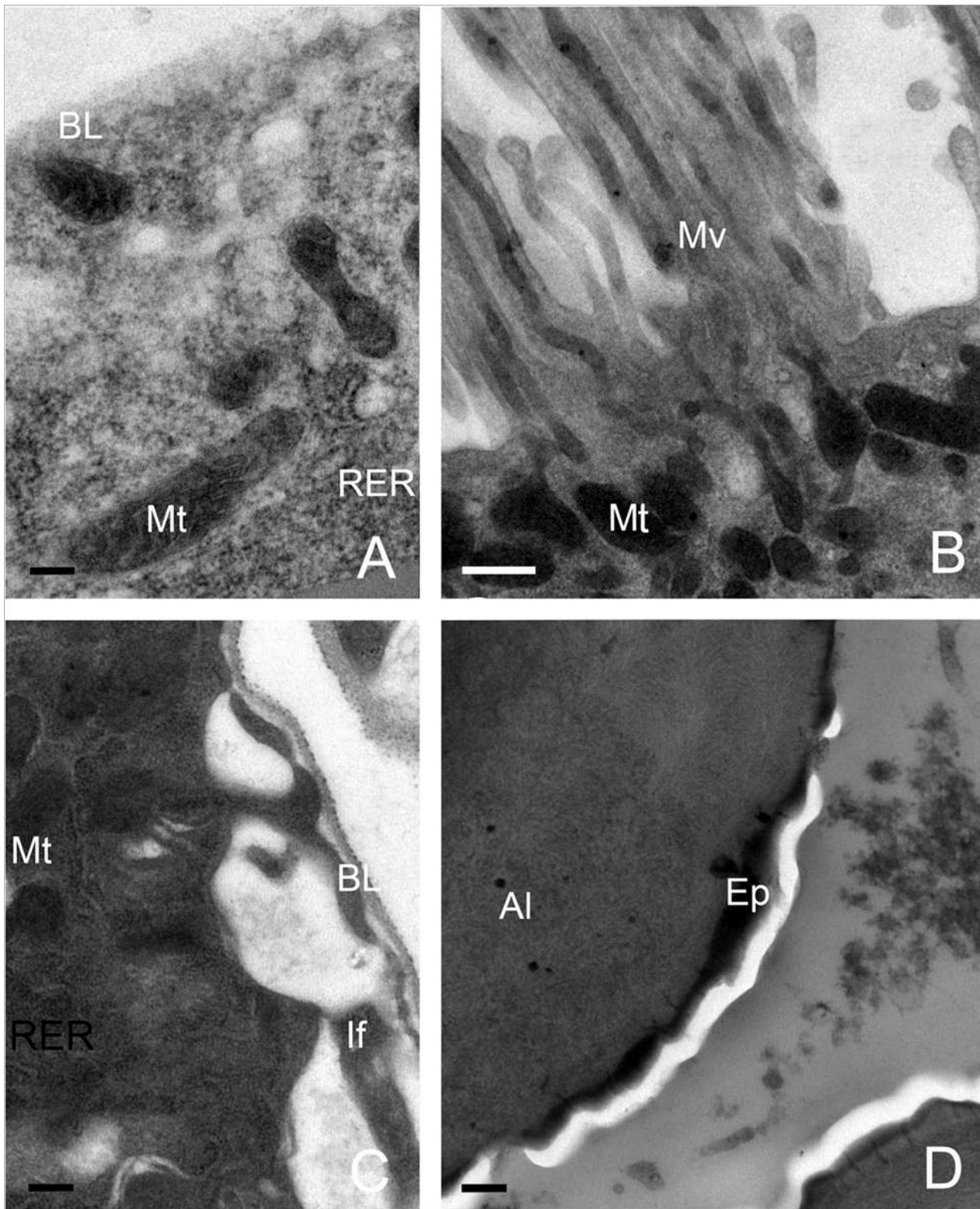


Figure 6

Electron micrographs of midgut cells of larvae of *C. suppressalis*. Scale bars: A, C and D 0.2 μm ; B 0.5 μm .

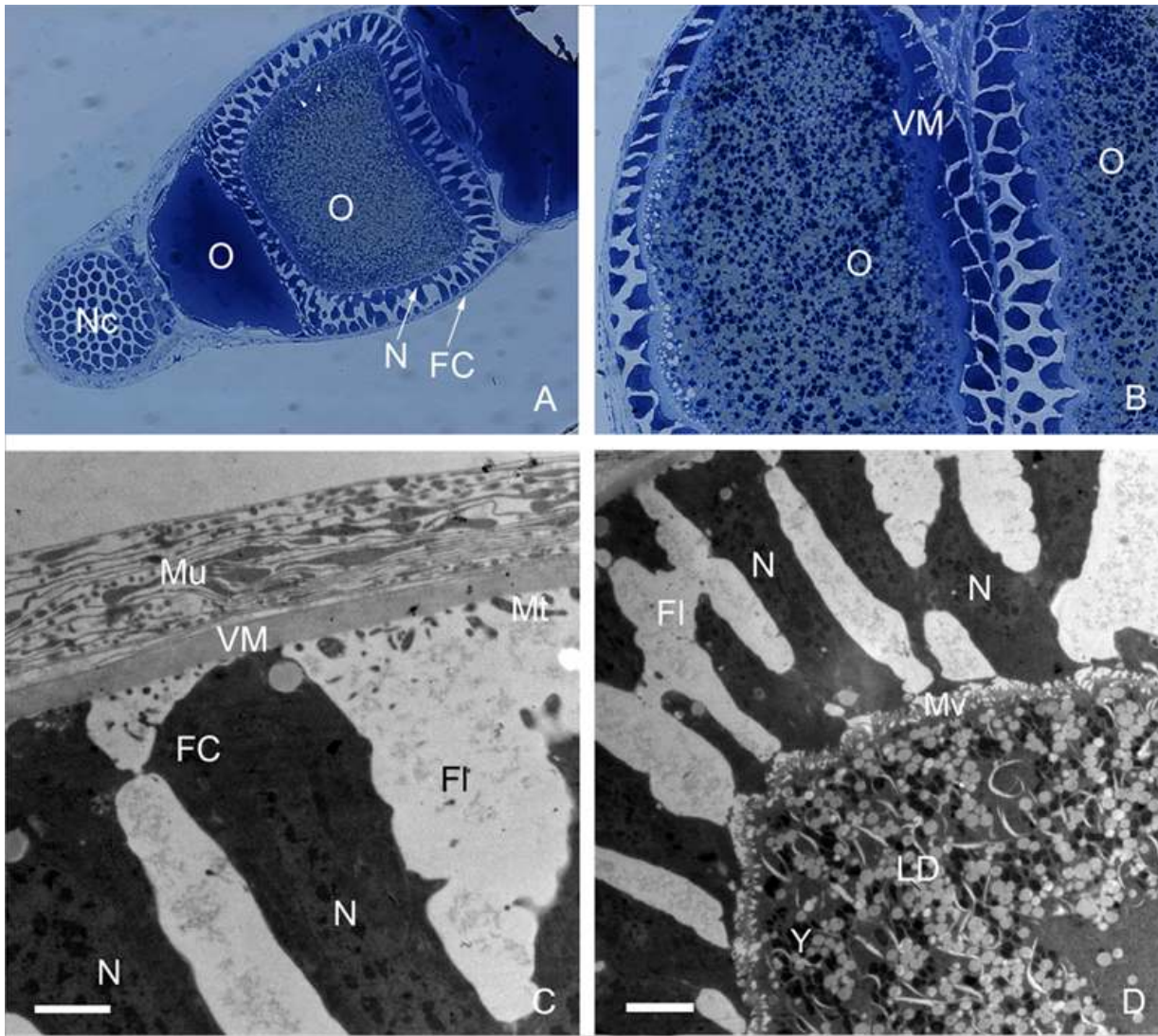


Figure 7

Electron micrographs of the ovary of female *C. suppressalis*. FC, follicle cell; FI, infoldings; LD, lipid droplets; Mt, mitochondria; Mu, muscles; N, nucleus; Nc, nurse cell; O, oocyte; VM, vitelline membrane; Y, yolk granules. Scale bars: C 2.0 μm ; D 5.0 μm .

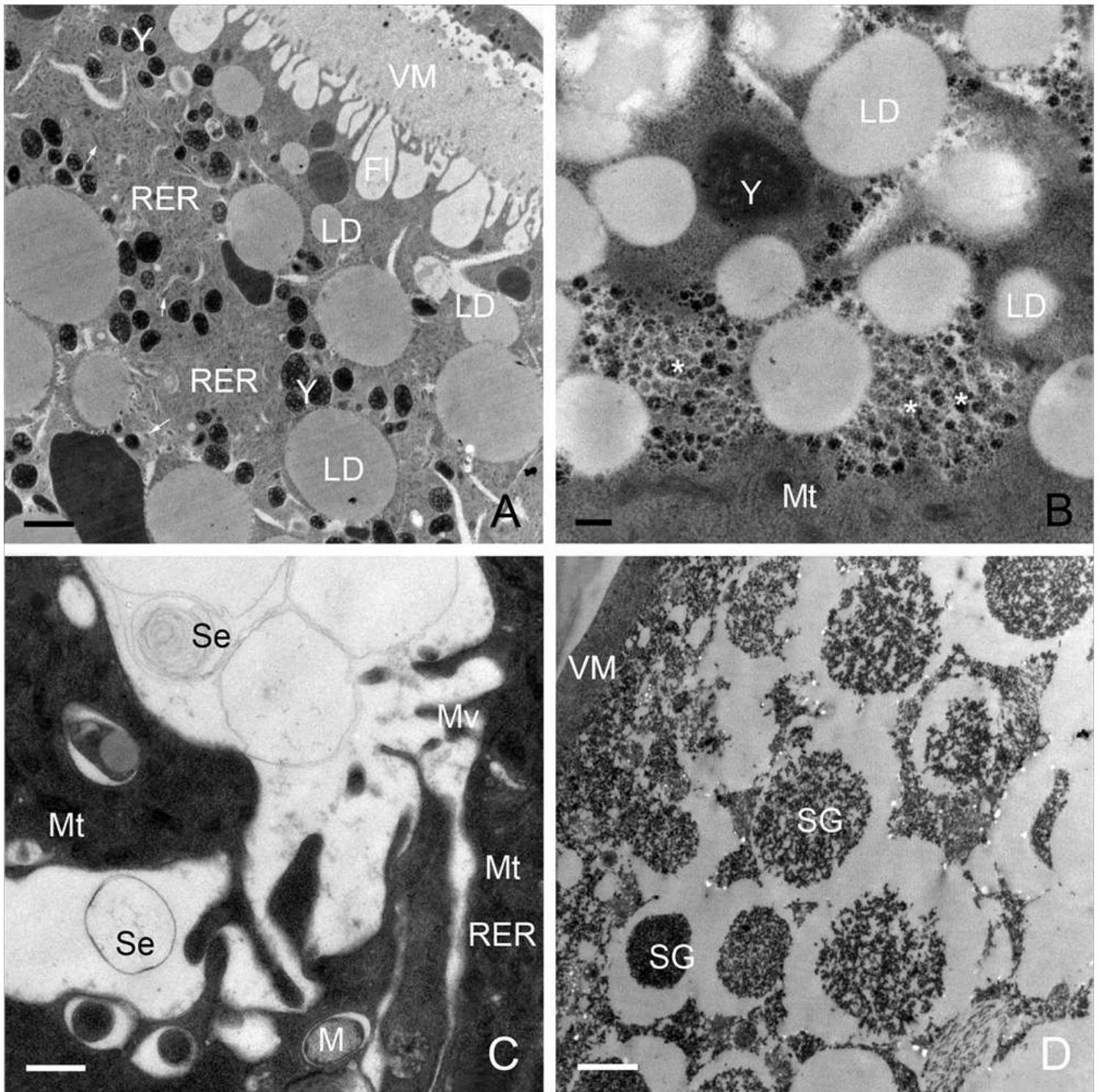


Figure 8

Electron micrographs of the ovary of female *C. suppressalis*. Fl, infoldings; LD, lipid droplets; Mt, mitochondria; RER, rough endoplasmic reticulum; Se, secretions; Sg, secretory granules; Y, yolk granules. Scale bars: A and D 2.0 μm ; B 0.2 μm ; C 0.5 μm .

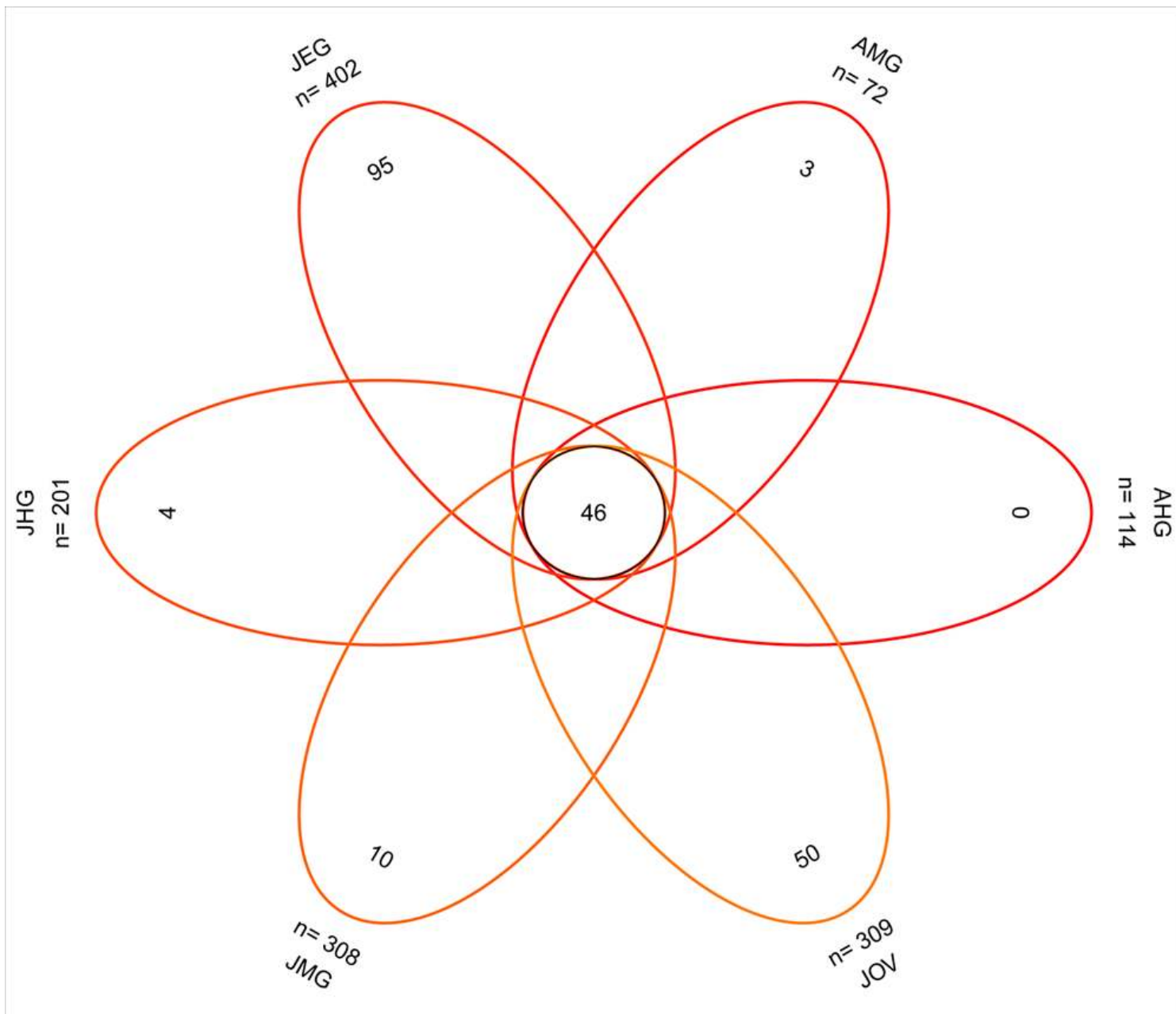


Figure 9

Venn diagram of OTUs from unique species owned by each sample and common species shared by two or more samples. AHG, hindguts of adult; AMG, midguts of adults; JHG, hindguts of larvae; JEG, eggslaid by females; JMG, midguts of larvae; JOV, ovary of females.

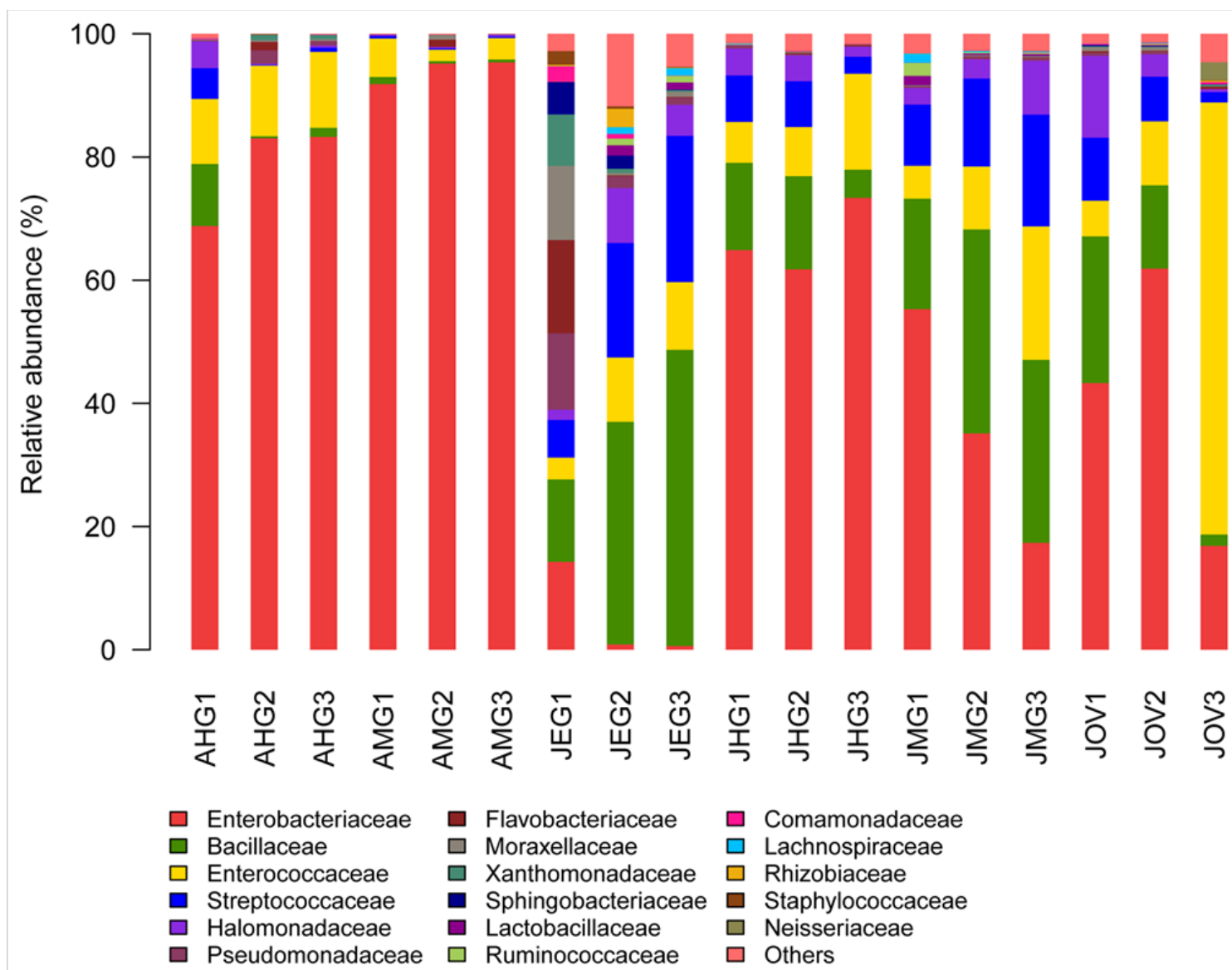


Figure 10

Compositions of gut microbiota at the family level across different developmental stages of *C. suppressalis*. The Y-axis represents the proportion of each taxon. Abbreviations: AHG1–AHG3, hindguts of adults; AMG1–AMG3, midguts of adults; JEG1–JEG3, eggs oviposited by females; JHG1–JHG3, hindguts of larvae; JMG1–JMG3, midguts of larvae; JOV1–JOV3, ovary of females.

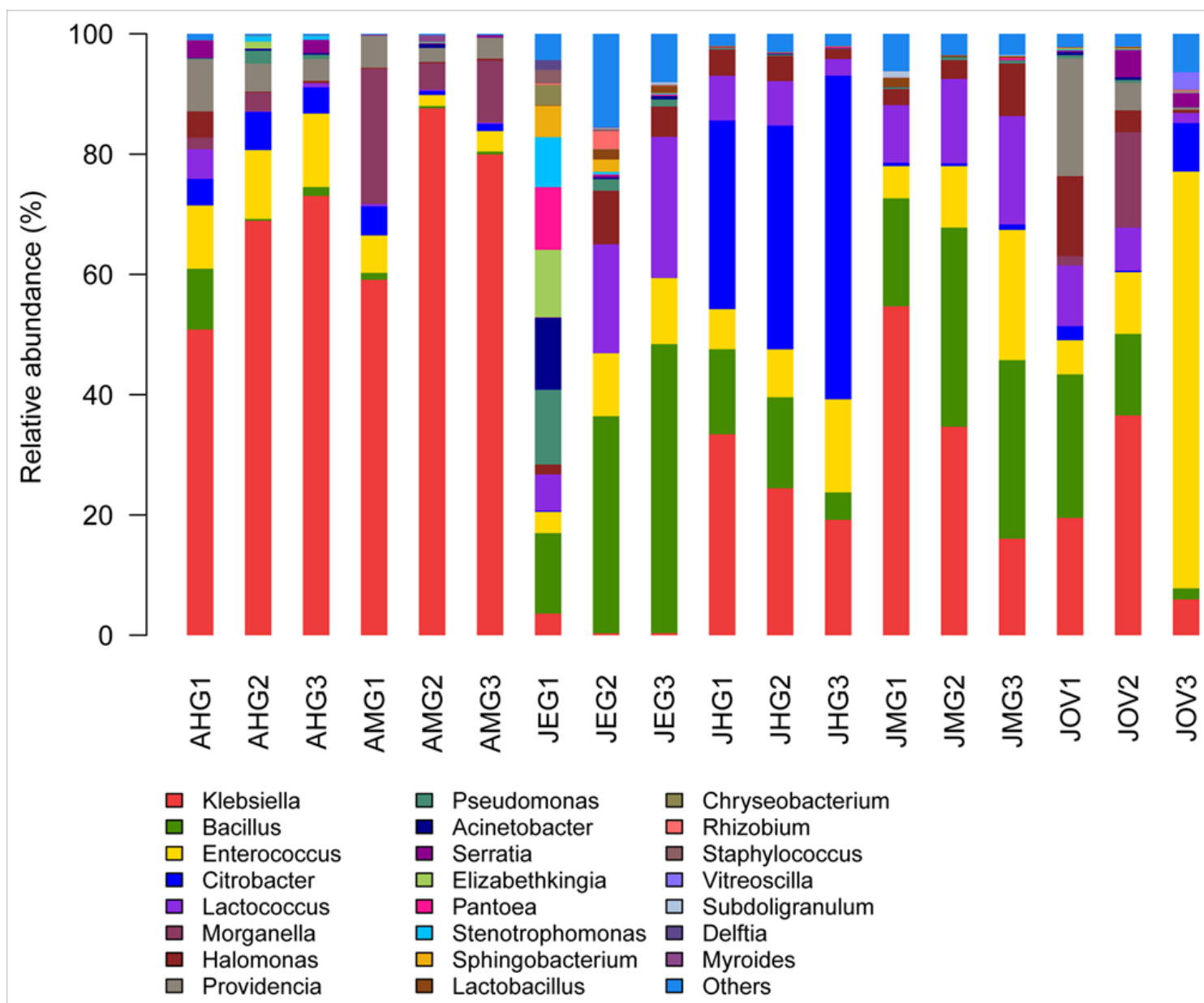


Figure 11

Compositions of gut microbiota at the genus level across different developmental stages of *C. suppressalis*. The composition of each sample was based on the taxonomic assignment of the 16S rDNA sequences. The Y-axis represented the proportion of each taxon. Abbreviations: AHG1–AHG3, hindguts of adults; AMG1–AMG3, midguts of adults; JEG1–JEG3, eggs oviposited by females; JHG1–JHG3, hindguts of larvae; JMG1–JMG3, midguts of larvae; JOV1–JOV3, ovary of females.

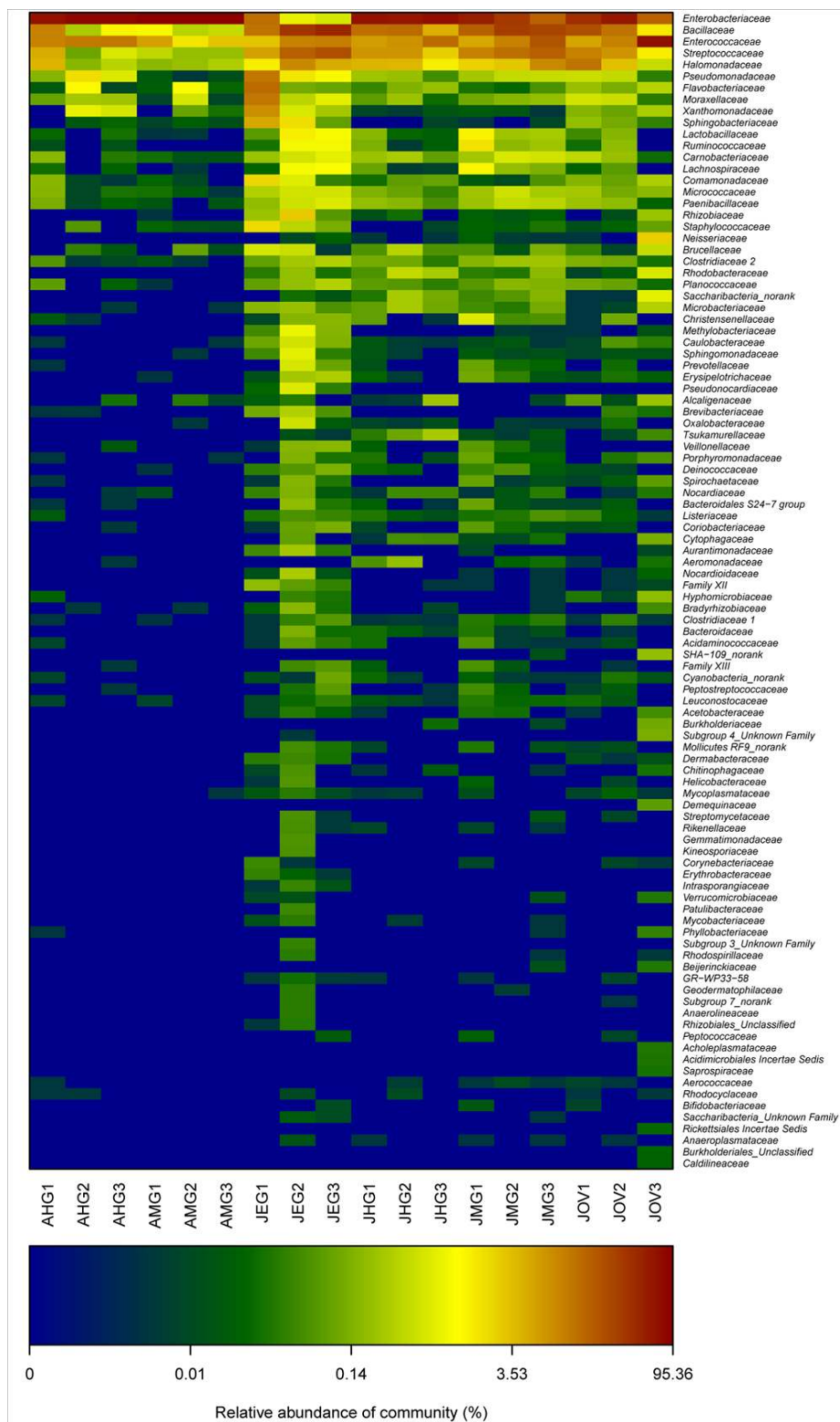


Figure 12

Abundance and composition of gut microbiota of all samples. Heatmap represent the proportions of OTUs at the family level. The X-coordinate represents the sample of each population, and the Y-coordinate represents the taxon. The color code indicates relative abundance, ranging from blue (low abundance) to yellow to red (high abundance). Abbreviations: AHG1–AHG3, hindguts of adults; AMG1–AMG3, midguts of adults; JEG1–JEG3, eggs oviposited by females; JHG1–JHG3, hindguts of larvae; JMG1–JMG3, midguts of larvae; JOV1–JOV3, ovary of females.

NMDS

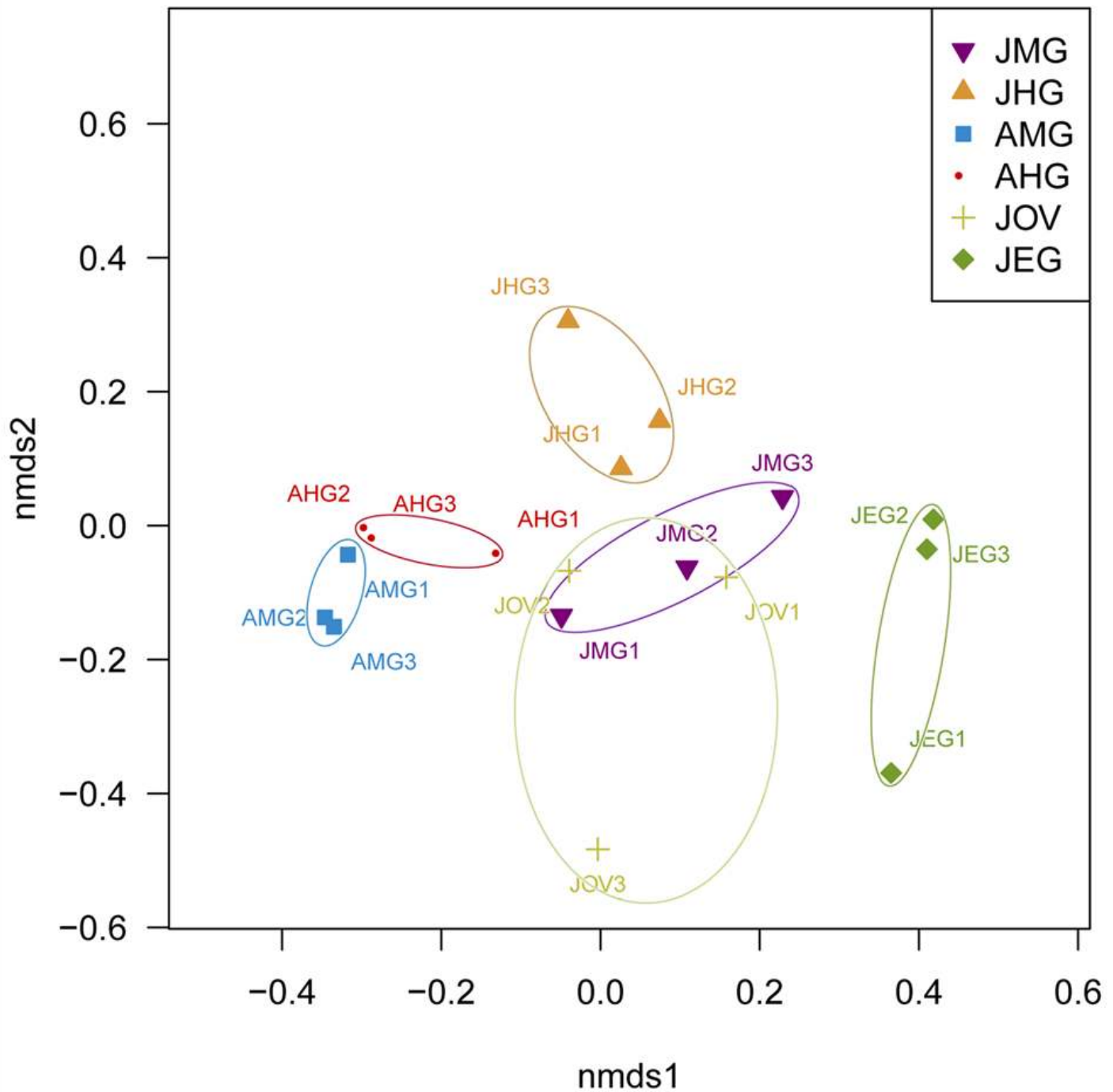


Figure 13

NMDS of the gut, ovary and egg microbiota of *C. suppressalis*. The samples were clustered by developmental stage, diet and compartment and represented with different colors: AHG (red, circles), AMG (blue, squares), JMG (purple, inverted triangle), JHG (safron yellow, triangle), JEG (green, rhombus) and JOV (yellow green, cross). The ellipses represent the standard error of the centroid for each group of samples with a confident limit of 95%. Abbreviations: AHG1–AHG3, hindguts of adults; AMG1–AMG3, midguts of adults; JEG1–JEG3, eggs oviposited by females; JHG1–JHG3, hindguts of larvae; JMG1–JMG3, midguts of larvae; JOV1–JOV3, ovary of females.

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