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# Alteration in microbes changed the contents of oviposition-deterrent pheromones on the *Spodoptera litura* egg surface

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## Abstract

Microorganisms symbiotic with insects, whether permanently or temporarily, play a crucial role in the nutrition, development, reproduction, defence, and metamorphosis regulation. In some Lepidoptera, oviposition-deterrent pheromones (ODPs) on egg surface were used by pregnant females to modify the behaviour of conspecifics to avoid excessive competition for limited resources. In this study, we constructed four different *Spodoptera litura* groups, including, OH, OA, SH, and OA, which either feed on different hosts or grow in different environments. The 16S rDNA libraries of microbes from the egg surface of the four groups were constructed and sequenced. According to *alpha* and *beta* diversity indices, the microbes in environments and diets considerably influenced the richness, diversity, and community compositions of the microbiota on egg surfaces. The quantity of the main ODP components and the corresponding oviposition-deterrent activity among four groups were significantly differed among the four groups. The result of this study revealed that altering of microbes in environments or diets considerably changed the contents of ODP and oviposition-deterrent activity. As ODPs impart oviposition-deterrent activity towards closely related species, the findings of this study suggest that we should pay more attention to the role of symbiotic microorganisms in changing the ability of insects, especially sympatric species, to occupy the optimal niche when developing novel pest-control strategies.

## Introduction

Pregnant females use oviposition-deterrent pheromones (ODPs) to modify the behaviour of conspecifics to avoid excessive competition for limited resources (Thompson and Pellmyr, 1991; Nufio and Papaj, 2001; Honda, 2010). Extracts from the egg masses of several Noctuid insects, including *Spodoptera littoralis*, *Mamestra configurata*, and *Helicoverpa armigera*, have been proved to have oviposition-deterrent effects on conspecific females (Ulmer *et al.*, 2003; Liu *et al.*, 2008; Gomaa, 2010). *Spodoptera litura* (Lepidoptera: Noctuidae) is a polyphagous pest that seriously threatens world agricultural production and food security (Ahmad *et al.*, 2013), whose preference for good-quality plants is weaker than that of oligophagous insects (Gripenberg, *et al.*, 2010). Pregnant *S. litura* presents a keen sensing ability to select optimal oviposition sites (Calumpang, 2013). However, whether the microbes in the environment and diet influenced the main ODP components and corresponding deterrent activity remain unclear.

Microorganisms symbiotic with insects, whether permanently or temporarily, play a crucial role in the nutrition, development, reproduction, defence, metamorphosis regulation, and numerous other functions in insects (Douglas, 2009; Provorov and Onishchuk, 2018; Malacrinò, 2022). Different insects require unique microbial populations in different environments, and sometimes even in different parts of the same insect, such as the salivary gland, midgut, and ovary, to perform unique physiological metabolism (Gimonneau *et al.*, 2014; Qiao *et al.*, 2019; Zhang *et al.*, 2023). For example, symbiotic microbes present in the gut of termites are essential for digesting cellulose and nitrogen required for nutrition throughout their life (Zhou *et al.*, 2019). Female *Costelytra zealandica* use phenol as their sex pheromone,

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which is produced by symbiotic bacteria in the accessory or colleterial gland (Marshall *et al*., 2016). The structure of insect bacterial communities is always shaped by various endogenous and exogenous factors, including parental transmission, environment, and host-associated bacteria (Wierz *et al*., 2021).

Many insects use vertical transmission via the egg as a mechanism to ensure the transfer of microorganisms that are added to the egg surface by the females in secretions or faeces (Paniagua Voirol et al., 2018). In particular, some neonate lepidopteran larvae bite through their egg shell while hatching and often fully ingest it after hatching, which will accelerate the spread of egg-surface microorganisms to the next generation (Brinkmann *et al*., 2008; Mason and Raffa, 2014).

Certain microorganisms associated with insects have been identified as the source of chemicals altering the behaviour of conspecifics or other organisms (Xu *et al*., 2016; Engl and Kaltenpoth, 2018; Mazorra-Alonso *et al*., 2021). *Drosophila* prefers to mate with conspecifics with a similar gut microbiota, which is probably linked to microbiota-dependent variation in the cuticle hydrocarbon profile (Sharon *et al*., 2011). Microorganisms play an important role in regulating metabolism, reproduction pheromone synthesis, and nutrition (Douglas, 2009; Provorov and Onishchuk, 2018; Xu *et al*., 2019). As an important kind of pheromone in insects, ODPs can avoid the fierce competition of offspring among the inter- or intra-specifics through altering the oviposition behaviour (Anderson and Löfqvist, 1996; Růžička, 1996), and microorganisms play an important role in regulating pheromone synthesis (Engl and Kaltenpoth, 2018; Mazorra-Alonso *et al*., 2021; Moyano *et al*., 2023). Therefore, exploring whether microorganisms in the host and environment can cause substantial variations in ODP components would provide important insights into the function of microorganisms in the competition among sympatric insects for host and ecological niches.

## Materials and methods

### Insect rearing and sample processing

The pupae of *S. litura* were originally purchased from Ke Yun Biological Liability Co., Ltd. The larvae were reared with the leaves of sweet potato and artificial diet, and the adults were offered 10% honey water. Composition (g) of the artificial diet was as follows: agar (3), soybean flour (15), wheatgerm (15), yeast powder (6.5), casein (3) methyl sorbate (0.375), multivitamins (0.15), ascorbic acid (0.6), and sodium benzoate (0.375), mixed in 150 ml of distilled water. The insectarium was kept at 27°C ± 2°C under 60–70% RH and an L12 (1200 lux):D12 h photoperiod (Gupta *et al*., 2005). To better understand the impact of the microbes on the host and environment in relation to the number of ODPs, the pupae were soaked in 75% alcohol for 55 s, 5–6 days after eclosion. The pupae were then rinsed three times with sterilised water in a super-clean environment. The water obtained from the third rinse was tested for sterility to ensure that the surface of the pupa was sterile. The surface-sterilised pupae were divided into four groups: OH, SH, SA, and OA. In the SH and SA groups, the surface-sterilised pupae, subsequent adults, eggs, and till to eggs of next generation were all continuously kept in sterile environment. In the SH and SA groups, the adults were both fed with sterilised honey water (10%), and the larval were reared with sterilised sweet potato leaves (75% alcohol 15 s, then 1% $NaClO_3$ 10 s, sterilised water three times) and sterilised artificial diet (103.4 kPa, 121.3°C, 15 min), respectively. Differently, the larvae in the OH group were kept in an open environment and fed with leaves of sweet potato collected directly from the outdoors; in the OA group, though the artificial diets were also sterilised, the whole insect breeding period was in an open environment.

### Library construction and high-throughput sequencing

Five hundred eggs from at least three different egg masses were collected and washed using 1 ml (1×) sterile phosphate buffered saline per sample using a sterile centrifuge tube. Each treatment had three replicates. The total genome DNA was extracted from the samples using the cetyltrimethylammonium bromide/sodium dodecyl sulphate method. The 16S rDNA genes were amplified in the hypervariable region of the bacterial 16S rDNA gene V4 region using the universal primer (F341: 5-CCTAYGGGRBG CASCAG-3; 806 R: 5-GGACTACNNGGGTATCTAAT-3) with a special barcode for each sample. Libraries were generated using Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) following purification with GeneJET Gel Extraction Kit (Thermo Scientific). The library quality was assessed using the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library sequencing was supported by Novogene Co., Ltd, China, on the Illumina NovaSeq PE250 platform. Paired-end reads from the original DNA fragments are merged using FLASH (Magoc and Salzberg, 2011) and filtered via the FASTP software package (Caporaso *et al*., 2010). The obtained effective tags, those with ≥97% similarity, were assigned to the same operational taxonomic units (OTUs) based on Uparse (Edgar, 2013). Then, a representative sequence was picked for each OTU, and RDP CLASSIfier was used to annotate taxonomic information based on the SILVA138 library via the Mothur method (Wang *et al*., 2007).

### Statistical and bioinformatics analysis

Alpha diversity indices, including PD whole tree, Ace, Shannon, and Simpson, were estimated using QIIME software (Version 1.9.1) and compared using R software based on Wilcox test to explore group variations (Dixon, 2003). Principal coordinates analysis (PCoA) based on weighted unifrac distance was conducted to illustrate the variability in the microbiota community composition between the groups. Furthermore, the significance of the four groups, including OH, SH, OA, and SH, was examined using the analysis of molecular variance (AMOVA) method based on weighted unifrac distance (Roewer, 1996). Based on the taxonomic information, the relative abundance of the top ten bacterial compositions of *S. litura* egg surface of four groups, including OH, SH, OA, and SH, at the phylum and genus levels. Furthermore, biomarkers with significant differences among groups at different taxonomic levels were identified considering four as the threshold of the linear discriminant analysis (LDA) score (Segata *et al*., 2011).

### Quantitative analysis of compounds eluted from the egg surface

Five hundred eggs of *S. litura* were collected 6–8 h after oviposition in a sterile environment. The eggs were subsequently placed in a 2 ml autosampler vial containing 0.5 ml *n*-hexane. After

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gentle shaking, the samples were stored at 4°C for 24 h. Next, 0.4 ml supernatant was collected into a new vial and stored at −20°C. Three biological replicates were performed. The extracts were analysed using Shimadzu GC-MS (QP2010 plus) equipped with a DB-WAXETR column (30 m × 0.25 mm ID, 0.25 µm film thickness). Helium was used as the carrier gas (1.0 ml $min^{-1}$). The temperature programme maintained an initial temperature of 110°C for 4 min, which was increased to 160°C at 10°C $min^{-1}$ and maintained for 1 min, and subsequently increased to 210°C at 4°C $min^{-1}$ and maintained for 3 min, and then increased to 240°C and maintained for 8 min. Using ethyl myristate as an internal standard compound, the quantity of six compounds that have been proved as the main ODP components, including palmitic, palmitoleic, stearic, oleic, linoleic, and α-linolenic acids, among the four groups was examined via one-way analysis of variance (ANOVA).

### Comparison of oviposition-deterrent activity

Newly emerged adults are placed in a mating cage, allowed to mate freely, and transferred to a new cage after mating to ensure each female has mated, who was placed in a cylinder (diameter: 9 cm; axial length: 16 cm) composed of metal mesh covered with filter paper (diameter: 12 cm) at each end. Compounds were mixed according to the ratio of the quantitative test and diluted in 0.3 ml hexane. The mixture was used as the control and coated evenly on the filter paper on one side of the cylinder cage. As the control, the filter paper on the opposite side was treated only with the same volume of hexane. During the bioassay, ventilation of the room should be turned on to vent odour continuously. Both ends of the test cage maintain the same light intensity. Ten replicates were carried out, and the number of eggs in each side were counted after 48 h. Oviposition-deterrent effect was indicated with oviposition-deterrent indices (ODI) (Lundgren, 1975). The ODI was calculated using the following equation: $ODI = (C - T)/(C + T)$, where $C$ is the number of eggs laid on the control, and $T$ represents the number of eggs on the treatment. The ODI was compared between the four groups with ANOVA.

## Results

### rRNA gene sequencing and microbiota comparison of the egg surface among the four groups based on α-diversity index

We obtained effective tags from each sample after merging and filtering the raw tags. The average number of clean tags obtained from the OH, SH, OA, and SA groups were 82,348, 84,204, 79,031, and 81,793, which represented 98.51, 98.37, 98.07 and 98.45% of the total number of raw tags, respectively. The index goods coverage was used to measure sequencing integrity. The coverage ranged from 99.80 to 100%, revealing that the majority of species present in the samples were well sequenced. Detailed information regarding library sequencing is provided in table 1.

The alpha diversity indices, including PD whole tree (fig. 1A), Ace (fig. 1B), Shannon (fig. 1C), and Simpson (fig. 1D) values, demonstrate the bacterial richness and diversity of the egg surface bacterial. Compared with the SH group, the OH group reveals higher PD whole tree, Shannon, and Simpson index values (Wilcox test, $P < 0.05$), which suggest that microorganisms in the environments considerably influence the richness and diversity of bacterial on the egg surface (fig. 1A, C, D). Although there is no significant difference in the Ace index between any two groups, group OH still shows higher bacterial diversity than the SH group (fig. 1B). The impact of environmental microorganisms on bacterial richness and diversity is also revealed by the extremely significant difference in the Simpson index between group OA and group SA (Wilcox test, $P < 0.01$) (fig. 1D).

When supplied with a sterile environment, the diet significantly influences the bacterial richness and diversity between the SH and SA groups, as per the Simpson index (Wilcox test, $P < 0.05$; fig. 1D). This suggests that the endophytic bacteria of the host play a decisive role in the structurally shaping egg surface microorganisms only in a sterile environment.

### Microbial community comparison of the egg surface among the four groups based on β-diversity index

A scatter plot was used to show the variability of microbiota communities among different groups based on weighted unifrac

**Table 1.** Quality summary of libraries construction and sequencing

| Sample name | Raw tags | Clean tags | Effective tags | Avg. length (nt) | Q30 | Goods_coverage |
|---|---|---|---|---|---|---|
| **OH1** | 90,270 | 88,839 | 74,819 | 426 | 93.78 | 0.998 |
| **OH1** | 83,591 | 82,368 | 72,098 | 428 | 93.3 | 0.998 |
| **OH1** | 76,922 | 75,836 | 70,616 | 429 | 93.69 | 0.999 |
| **SH1** | 87,370 | 86,200 | 80,070 | 429 | 93.65 | 0.999 |
| **SH1** | 88,661 | 87,123 | 79,538 | 428 | 93.04 | 0.999 |
| **SH1** | 80,776 | 79,289 | 73,095 | 428 | 93.23 | 0.998 |
| **OA1** | 77,959 | 76,262 | 69,352 | 428 | 92.96 | 0.999 |
| **OA1** | 78,967 | 77,526 | 70,680 | 428 | 93.5 | 0.999 |
| **OA1** | 84,833 | 83,304 | 75,308 | 427 | 93.25 | 0.999 |
| **SA1** | 82,370 | 81,133 | 71,012 | 427 | 93.42 | 1 |
| **SA2** | 85,526 | 84,061 | 75,421 | 428 | 93.2 | 0.999 |
| **SA3** | 81,342 | 80,185 | 70,981 | 428 | 93.88 | 0.999 |

OH, group provided with host in open environment; SH, group provided with sterile host and environment; OA, group provided with artificial diets in open environment; SA, group provided with artificial diets in open environment.

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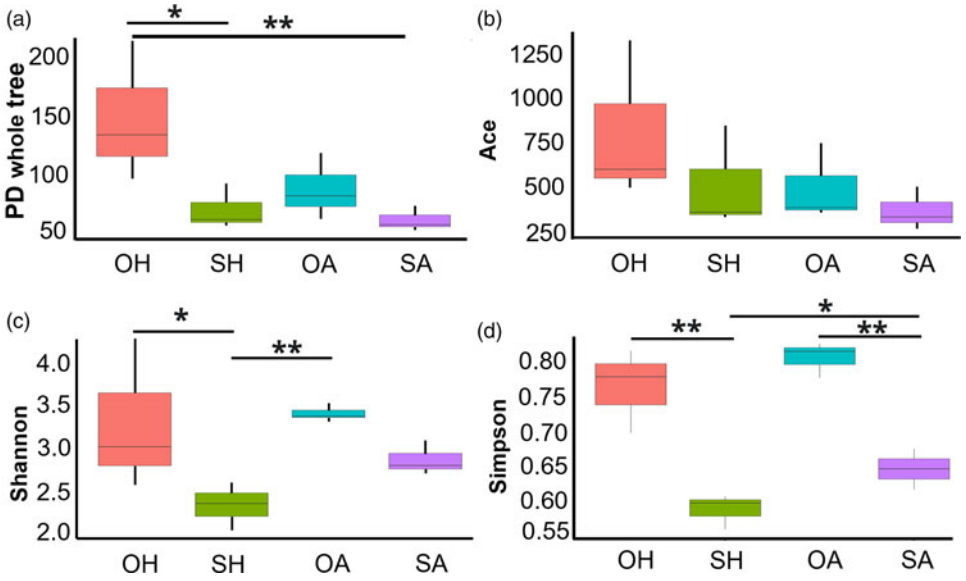


**Figure 1.** Comparison of alpha diversity index, including (A) PD whole tree, (B) Ace (C) Shannon, and (D) Simpson values, of microbial communities on the egg surface of *S. litura* supplied with four diet or environment. Alpha diversity index of each group was compared with Wilcox test. *$P < 0.05$; **$P < 0.01$.

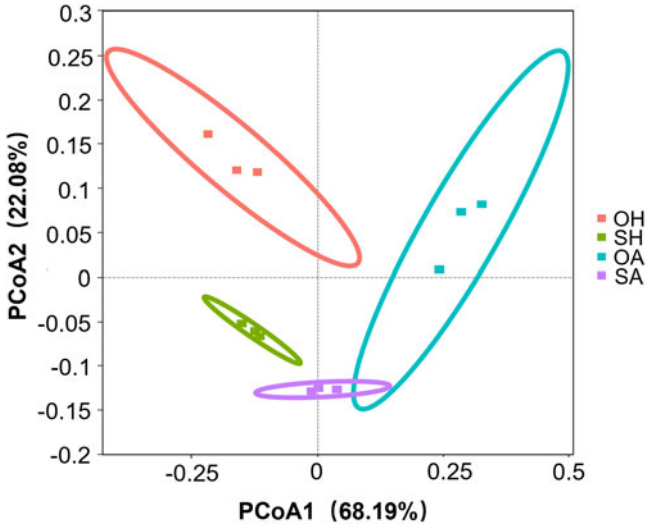


**Figure 2.** PCoA for microbiota variability based on weighted unifrac distance. Each symbol represents a sample. The variance explained by the PCoA is indicated on the axes.

distance (fig. 2). The first two principal coordinates of the plot, PCoA1 and PCoA2, accounted for 68.19 and 22.08% of the data variation, respectively. The AMOVA analysis revealed a significant difference among the four groups ($P < 0.001$).

The taxonomic information of each OTU at different levels was obtained according to the annotation result in the SILVA138 library. Figure 3A, B show the relative abundance of the top ten bacterial compositions found on the surface of *S. litura* eggs of the OH, SH, OA, and SA groups at the phylum and genus levels, respectively. OTUs that were not assigned to known microbial phyla were designated as 'Unidentified Bacteria', which, on average, accounted for 0.69% of the total data (fig. 3A). Microbial phyla with low abundance were grouped together as 'others'. The Proteobacteria (mean ± SD = 82.9649 ± 15.32%) was the main microbial phyla of all the samples. However, the relative abundance of Firmicutes phylum in the groups fed with artificial diet, i.e. OA (37.82 ± 5.33%) and SA (11.38 ± 1.32%), was significantly higher than that in the host-supplied groups OH (2.59 ± 0.66%) and SH (1.73 ± 1.02%; ANOVA, Duncan, $P < 0.05$; fig. 3A). At the genus level (fig. 3B), the major genera found in the samples changed significantly depending on the environment or diet. Acinetobacter (54.96 ± 13.48%) in the OH groups, Enterobacter (74.55 ± 1.97%) in the SH groups, Enterococcus (34.60 ± 5.24%) in the OA groups, and Providencia (58.56 ± 2.68%) in the SA groups were significantly higher than that in the other groups (ANOVA, Duncan, $P < 0.05$).

To identify the biomarker that revealed significant differences among the groups at different taxonomic levels, LDA was conducted using four as the threshold (fig. 4). At the genus level, some new genera were identified as the biomarker. For example, Enterococcus and Serratia genera were identified as the dominant genus and important biomarker of the OA group, respectively. Similarly, except for Providencia, Tazewell was also identified as the biomarker of the SA group.

### Comparison of ODP components among the four groups

Figure 5 shows a decreasing trend in the average levels of six compounds from the OH, SH, and OA groups to the SA group. The OH and SH groups were fed on the host; however, the OH group exhibited significantly higher levels of palmitoleic, stearic, oleic, and linoleic acids compared with the SH group. When supplied with an artificial diet, the levels of palmitic and α-linolenic acids in the SA group were significantly lower than those in the OA group. The comparison between the OH and OA groups, both of which were supplied in open environments, also revealed significant differences in the levels of four components, including palmitoleic, stearic, oleic, and linoleic acids. In a sterile environment, replacing sweet potato leaves with an artificial diet significantly decreased the levels of stearic, oleic, and α-linolenic acids.

### Oviposition-deterrent activity assay and comparison

Compounds were mixed according to the ratio of the quantitative test and the ODI among the four groups was compared, and the result revealed that the ODI of the OH group was significantly

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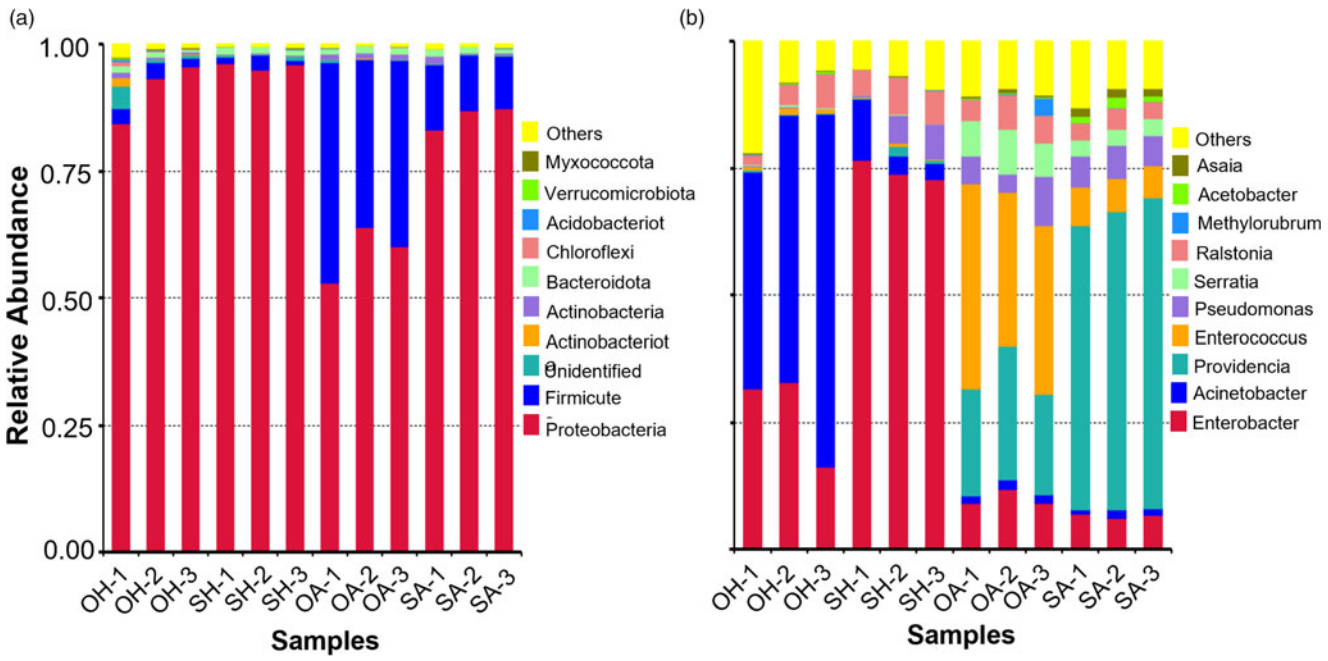


**Figure 3.** Relative abundance of top ten bacterial composition of *S. litura* egg surface supplied with four different diets, including OH, SH, OA, and SH at the phylum at (A) the phylum and (B) genus levels, respectively.

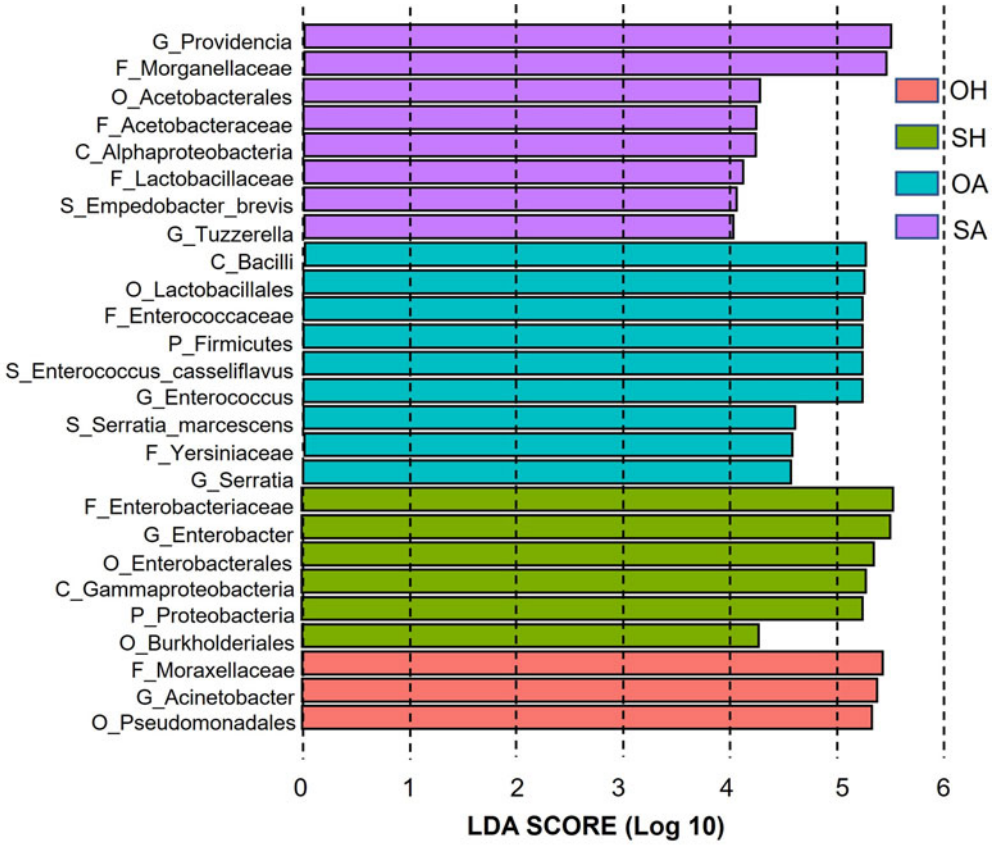


**Figure 4.** Identification of the bacterial biomarker among the groups at different taxonomic levels; LDA was conducted using four as the threshold. The means of capital letter before the name of biomarkers is phylum (P), class (C), order (O), family (F), genus (G), and species (S).

higher than that of the SH, OA, and SA groups. Comparably, the ODI of the SA group was significantly lower than that of other groups. There was no obvious difference between the SH and OA groups (fig. 6). These results suggest that changes in the microbial population in environments or diet considerably affect the levels of compounds on the *S. litura* egg surface.

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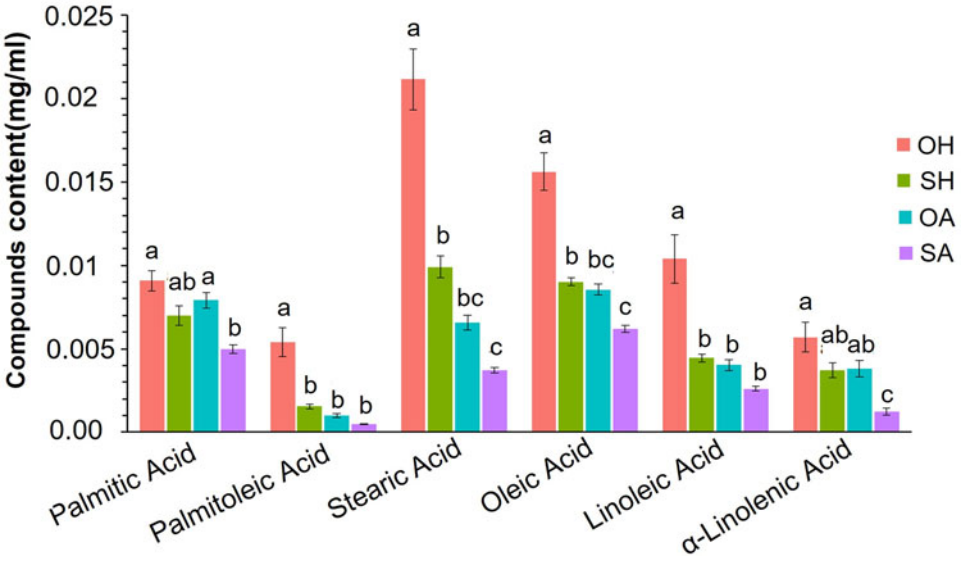


**Figure 5.** Comparison of ODP contents from the egg surface of *S. litura* provides different diet or environment. Different letters indicate a significant difference in the relative expression levels (one-way ANOVA, $P < 0.05$). OH: group provided with natural host in open environment; SH: group provided with sterile host and environment; OA: group provided with artificial diets in open environment; SA, group provided with artificial diets in open environment.

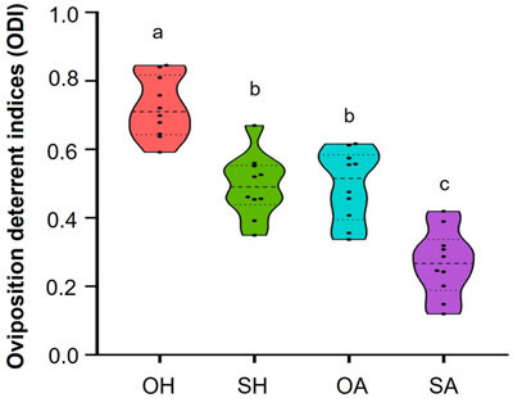


**Figure 6.** Comparison of oviposition deterrent activity of ODPs composed of authentic compounds equivalent to 500 eggs among the OH, OA, SH, and SA groups. Different letters indicate a significant difference in the relative expression levels (one-way ANOVA, $P < 0.01$).

## Discussion

Microorganisms supply the necessary assistance to insects in different habitats to complete their unique biology, development, fitness, and lifestyle processes (Kellner, 2003; Douglas, 2009; Colman *et al.*, 2012). Phytophagous insects harbour symbiotic bacteria that can be transmitted vertically from parents to offspring, in which the egg is a key link between stages of completely different morphologies.

The specialised structure of the terminal digestive tracts and genital ensures that symbionts are transmitted from the gut to the outside of the eggs (Kellner, 2003). A recent study using 4000 publicly available sequencing data found that besides taxonomy, the origin of the sample (whether from a laboratory or fields) (Shannon $R^2 = 0.087$; $P < 0.001$) and the diet (Shannon $R^2 = 0.036$; $P < 0.001$) were important factors that affected variation in the bacterial diversity of the gut lumen (Malacrinò, 2022). Herein, we compared the richness and diversity of microbiota at the egg surface of *S. litura* supplied with different environments and diets. Compared to diets, environmental factors had a greater influence on bacterial richness and diversity, which agrees with the results of large-scale data analysis of guts.

In the guts of insects from different orders, the dominant microbial phylum was identified as the Proteobacteria or/and Firmicutes phyla (Dillon and Charnley, 2002; Haynes *et al.*, 2003; Schloss *et al.*, 2006; Behar *et al.*, 2008; Li *et al.*, 2022). At the genus level, environment and diet greatly influenced the microbial structure of the *S. litura* guts (Xia *et al.*, 2020). Similar to that in the guts, the result of this study showed that the microbial structure of the egg surface could be influenced by microbes in the environments and diets.

Microorganisms associated with eggs may produce antibiotics that prevent the new hatching larvae from pathogen infection, implying that microbe variations on the egg surface may play an important role in environment adaptability. For example, bacteria on the egg surface of *Holotrichia oblita* inhibit the multiplication of the entomopathogens *Bacillus thuringiensis* and *Beauveria bassiana* (Wang *et al.*, 2021). ODPs were used by pregnant females to avoid excessive competition for limited resources. The same ODP components shared among closely related species can produce oviposition-deterrent activity to closely related species. For example, a mixture of the five fatty acids found in *Ostrinia zealis* exhibited significant oviposition-deterrent effects on all the three other *Ostrinia* species (Li and Ishikawa, 2004). Herein, *S. litura* ODPs were identified, and the main components were fatty acids, some of which were shared with other lepidopteran insects, such as *Spodoptera frugiperda*, *H. armigera* (Li *et al.*, 2001), *Ostrinia furnacalis* (Li and Ishikawa, 2004), and *Phthorimaea operculella* (Zhang *et al.*, 2018). The relationship between the relative levels of unsaturated fatty acids in eggs and microorganisms was reported in an early study (Jackson *et al.*, 1968). The results of this study revealed that the quantity of ODPs and the deterrent activity will be influenced by the microorganisms depending on environments and diets. ODP component changes may cause alteration in the abilities of intraspecific and interspecific insects to occupy the most suitable niches. Chemical substances present

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on the egg surface and egg-associated microorganisms could trigger downstream defence responses in the host (Hilfiker *et al.*, 2014; Bertea *et al.*, 2020; Li *et al.*, 2023), and the induced plant volatiles could be used as synomones in attracting natural enemies (Fatouros *et al.*, 2008, 2005; Conti *et al.*, 2010). The findings of this study suggest that we should pay more attention to the role of microorganisms in changing the ability of insects, especially sympatric species, to occupy the optimal niche when developing novel pest-control strategies.

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**Author contributions.** Jianmei Shen and Zhenfei Zhang designed the study; Liming Hu wrote the manuscript; Yirui Chen mainly performed quantitative analysis of compounds; and all authors have equally important contributions in other instrument operation and data analysis.

**Competing interests.** None.

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