

研究报告

## Symbiotic *Escherichia coli* promotes the developmental timing of *Drosophila melanogaster*

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**Abstract:** [Background] The symbiotic microbiota profoundly affects many aspects of host physiology, but the diversity and complexity of microbial community make it difficult to explore the underlying mechanism in vertebrates. Fruit fly *Drosophila* provides us a germ-free and gnotobiotic model to investigate the interaction of microbes and hosts. [Objective] To isolate and identify *Escherichia coli* from *Drosophila melanogaster* gut and investigate the effects of *E. coli* on the development of hosts. [Methods] *E. coli* was isolated with selective medium and identified with BLASTn analysis of 16S rRNA gene. *In vitro* and *in vivo* co-existence test were used to verify the symbiosis. Through the developmental timing and growth rate experiments, the effect of *E. coli* on hosts' development were investigated. Real-time quantitative PCR were used to assess gene expression levels of *PTTH* and insulin signaling pathways. [Results] We isolated and identified indigenous strains of *E. coli* in the guts of both lab-reared and wild-captured *Drosophila*. *E. coli* was co-cultured with commensal *Lactobacillus plantarum* *in vitro*, and *in vivo* colonized the fly gut, indicating that *E. coli* was one symbiotic member of the bacterial community of *Drosophila*. Moreover, *E. coli* facilitated the development of *Drosophila* by accelerating the growth rate. At the molecular level, *E. coli* significantly stimulated the activity of *PTTH* and insulin signaling that is essential for the larval/pupal transmission in *Drosophila*. [Conclusion] *E. coli* was symbiotic bacteria of *Drosophila* and promoted the development of *Drosophila*.

**Keywords:** *Escherichia coli*, *Drosophila*, Symbiosis, Growth and Development, Insulin signal pathway

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# 共生大肠杆菌促进黑腹果蝇生长发育

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**摘要:**【背景】共生菌对宿主的很多生理功能有着重要影响,但微生物菌群的多样性和复杂性使得探索其潜在的机制存在困难。黑腹果蝇的无菌和悉菌模型可以被用来研究细菌和宿主的相互作用。【目的】分离和鉴定果蝇肠道大肠杆菌,并研究其对宿主生长发育的影响。【方法】利用大肠杆菌选择性培养基分离果蝇肠道大肠杆菌,通过16S rRNA基因序列比对鉴定菌株。利用体外和体内定殖实验验证共生关系。通过果蝇的发育历期和生长速率实验检测该细菌对宿主生长发育的影响。利用RT-qPCR技术对促胸腺激素及胰岛素信号通路相关基因的表达水平进行检测。【结果】从实验室饲养和野生果蝇肠道体内分离并鉴定得到大肠杆菌。体内和体外定殖试验中大肠杆菌可以和果蝇肠道共生菌共存,说明大肠杆菌是果蝇肠道共生菌。另外,大肠杆菌通过提高果蝇生长速率促进其发育。在分子水平上,大肠杆菌可以激活果蝇体内脑促胸腺激素和胰岛素信号通路相关基因的表达。【结论】大肠杆菌是果蝇肠道共生菌并能促进果蝇生长发育。

**关键词:** 大肠杆菌, 黑腹果蝇, 共生, 生长发育, 胰岛素信号通路

## 1 Introduction

Metazoan guts act as a natural cabinet, where microbiota and environmental factors constitute an open and dynamic intestinal microecological system. The dynamic balance of bacteria and metabolites generated from microbial transformation of diet has important influences on physiological function of hosts<sup>[1]</sup>. For instance, studies have demonstrated that spore-forming bacteria and their metabolites, 5-HT, significantly impact host physiology by modulating gastrointestinal motility and platelet function<sup>[2]</sup>. In addition, the gut microbiota restricted the colonization of pathogens in host by promoting proper immune system development and local immune homeostasis<sup>[3]</sup>. Inversely, the imbalance of intestinal microecological system causes many diseases, such as colorectal cancer, nervous system disease, depression, and obesity<sup>[4-6]</sup>.

Over the last two decades, the fruit fly *Drosophila* has been largely used to decipher the competitive interaction of hosts and microbes<sup>[7]</sup>. Recent studies have suggested the application of this model in elucidating the underpinning mechanisms of commensal host-gut microbiota interactions, due to its

amenability to genetic study, lower microbiota complexity, and the ease in manipulating germ free (GF) flies<sup>[8]</sup>. Studies showed that laboratory stocks were associated with a relatively low number of taxa, and were frequently restricted to two genera, *Acetobacter orientalis* and *Lactobacillus plantarum*<sup>[8]</sup>. However, *Drosophila* is saprophytic and mainly feeds on decaying fruits with an abundance of fermenting microbes, so it was assumed that wild flies encounter a greater diversity of microbes in natural environment than in the laboratory. As such, this provides the opportunity for us to uncover the diversity and functions of microbial community.

As one of the best-characterized model organisms, *E. coli* was thought to be mainly an inhabitant of the intestines and faeces of warm-blooded animals<sup>[9-10]</sup>, totally consisting of more than 500 species and approaching the density of  $10^{10}$ – $10^{11}$  cells/g in large-intestinal content<sup>[11]</sup>. However, *E. coli* transits in water and sediment, which usually contaminate food. The decaying food provides a resource of microbes as well as nutrition for saprophytic animals, like fruit flies. During ingestion, *Drosophila* acquires polymicrobial mixtures of bacteria in a great variety of

habitats, and sustained their microbial gut community by frequent replenishment of them<sup>[12]</sup>. Given that many bacteria species remain uncovered, we proposed that *E. coli* could be symbiotic bacterium among *Drosophila* populations.

Here, we reported that *E. coli* was isolated from both laboratory-reared fly stocks and wild-fly stocks. Colonization in parental generation and progeny of *E. coli* defined the symbiotic bacteria of *Drosophila*. Moreover, *E. coli* stimulated the development of germ-free *Drosophila* by accelerating *Drosophila* growth rate and stimulating the expression of Prothoracicotropic hormone (*PTTH*) and *InR* gene. Our results revealed that *E. coli* could be one of integral contributors to the development of *Drosophila*, providing an insight into the excellent model of *E. coli* and *Drosophila* in the future.

## 2 Materials and Methods

### 2.1 Fly stocks and rearing

*Oregon R* flies were used as wild type stocks. Wild fly stocks were captured with rotten fruits as baits in the field in Guangzhou (GZ), Xi'an (XA), Liaoning (LN) and Fenyang (FY) of China. *Drosophila melanogaster* were maintained at 25 °C, 50% relative humidity, under a 12 h/12 h light/dark cycle in the incubators. Flies were cultured with standard food medium (77.70 g maize flour, 24.00 g yeast, 0.83 g CaCl<sub>2</sub>, 31.60 g sucrose, 63.20 g glucose, 18.00 g agar, 8.80 g potassium sodium tartrate tetrahydrate, 1 350 mL H<sub>2</sub>O and 14.7 mL 10% n-Butyl-p-hydroxybenzoate), unless otherwise noted. Fresh food was cooked for 10 min in boiling water and prepared every week to avoid desiccation. Conventional reared (CR) flies were raised as usual, while wild fly stocks were reared on autoclaved fly medium to avoid the contamination of laboratory bacteria. The cornmeal-casein medium (10.0 g agar, 70.0 g cornmeal flour, 1.0, 5.0 or 20.0 g casein, 50.0 g sucrose and 1 L H<sub>2</sub>O) was used to study the timing of development in *Drosophila*, and the yeast/cornmeal medium (15.0 g agar, 70.0 g cornmeal flour, 5.0 g yeast, 50.0 g sucrose and 1 L H<sub>2</sub>O) was for colonization and growth ration assay.

### 2.2 Preparation of GF, gnotobiotic and CR flies

To produce axenic pupae and flies, we collected freshly laid-eggs within 10 h into 1.50 mL Eppendorf (EP) tube from grape juice agar plates. First, embryos

were sterilized with diluted Walch (1:30) whose effective component is 4-chloro-3,5-xyleneol for three times, then treated with diluted hypochlorite (1:1, Sigma), and finally washed twice with 70% ethanol. The sterile embryos were washed with 0.01% PBST (PBS solution with Triton X-100) until there was no bleach smell. The embryos were aseptically transferred to autoclaved media. Germ-free eggs were ascertained by the column-forming-units (CFU) of bacteria on nutrient agar with the ground embryos. For gnotobiotic fly preparation, 1 mL of bacteria medium with 1 OD value were centrifuged for 1 min at the speed of 4 000 r/min, and then the supernatant was discarded. Bacterial cells were washed with sterile 1 mL PBS, and were centrifuged for 1 min at the speed of 4 000 r/min followed by supernatant removal. The pellets of bacteria (*E. coli*, *L. plantarum*) were suspended with 50 µL PBS, and the mixed inoculum was added to the surface of autoclaved food in vials sealed with plugs. To avoid contamination with other microbes, the vials were maintained in a sterile cell culture hood until the flies reached the adult stage. For CR sibling flies, embryos without disinfection were directly transferred to sterile food.

### 2.3 Bacterial isolation and identification

Guts of samples of wild-captured and lab-reared flies were dissected and transferred to the PBS solution on ice. Guts were washed with 70% ethanol solution and sterile PBS, and homogenized by motorized pestle in PBS. The homogenates were plated immediately on MAC agar to isolate the *E. coli* of the commensal flora. Plates were incubated at 37 °C in the incubator overnight. Single colonies were transferred to YCFA broth plus 0.25% glucose (YCFAG) for 12 h at 37 °C<sup>[13]</sup>. DNA extraction and amplification were carried out as described before commercial sequencing<sup>[14]</sup>. Phylogenetic analysis was based on the sequences of 16S rRNA gene (Primer set: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3'<sup>[14]</sup>), of isolated bacteria and related ones downloaded from GenBank. Phylogenetic tree was constructed by the Neighbor-Joining method with MEGA 6.0 software.

### 2.4 Co-cultivation of *E. coli* and *L. plantarum* *in vitro*

*E. coli* and *L. plantarum* strains were recovered and activated twice prior to the fermentation

experiments. *E. coli* strains were inoculated in a roll tube containing 10 mL sterilized YCFAG broth, and then cultured at 37 °C overnight in the incubator to grow at the density of 1 OD value. For *L. plantarum*, they were inoculated in MRS broth. The liquid of 2 mL *E. coli*, 2 mL *L. plantarum* and 2 mL mixture (1 mL of each strain) were inoculated into the fermenting bottles containing 80 mL of YCFAG broth, respectively. Three repeats of each group were performed, and then cultured at 37 °C in the incubator. The OD and pH values of bacteria suspension were determined with spectrophotometer (Ultraviolet spectrophotometry 1 800) and PB-10 acidometer in time course. Number of each bacterium was assessed with CFU on specific medium, MAC Agar (AOBOX) for *E. coli* and MRS (AOBOX) Agar for *L. plantarum*.

## 2.5 Bacterial load analysis

Bacterial load of surface-decontaminated individuals was quantified by plating serially diluted lysates of 10 individuals (larvae, pupae or adults) on MAC agar plates (MRS for *L. plantarum*). In brief, the surface-decontaminated individuals were obtained by surface-sterilizing flies twice in 70% ethonal solution with agitation, and then rinsed twice in sterile water. Individuals are transferred to an EP tube carrying 0.20 mL of sterile PBS and homogenised with a micropestle. The bacterial load of fly medium was performed by dissolving 0.10 g food into 1 mL of sterile PBS. Lysates and fly medium with proper dilution were evenly deposited on the surface of MAC agar medium and then incubated at 37 °C in the incubator overnight.

## 2.6 Developmental timing measurements and larval size measurements

The number of pupa formation and adult emergency was counted to assay developmental timing of individuals over time. For the measurement of larval size, 10 individuals were collected and froze in freezer everyday until the emergence of pupae in the yeast/cornmeal medium. Dead larvae were mounted with ddH<sub>2</sub>O, and pictures were taken on a black background using stereoscopic microscope. Body area of each larval surface was calculated using ImageJ, and the area of body surface was showed with pixel value.

## 2.7 Transfer of *E. coli* in *Drosophila* between generations

The pupae in gnotobiotic group was disinfected twice with 70% ethanol before transferred to a new sterile medium. Moving away the 2 d adult and the number of bacteria per gut in filial fly were assessed.

## 2.8 RNA extraction and reverse transcription

Ten individuals of CR, GF and *E. coli*-associated flies, ranging from day 3 after egg laying (AEL) to day 3 after pupa emergence, were obtained from 0.5% yeast/cornmeal medium. Total RNA from three biological replicates was extracted by Trizol method (Invitrogen), template RNA (2 µg) was used to generate cDNA by reverse transcription with oligo-dT, followed by analysis by Real-time quantitative PCR (RT-qPCR) using a instrument (Bio-Rad) and the SYBR Green (TaqMan).

## 2.9 RT-qPCR

We used the  $\Delta C_t$  method for data analysis, using *rp49* as the reference gene. The relative levels of given mRNA was calculated according to cycling threshold analysis:  $\Delta C_t = C_t(\text{target gene}) - C_t(\text{reference gene})$ , the relative =  $2^{-\Delta \Delta C_t}$ . Primer set for *PTTH* gene (F: 5'-CACTCCACATCCCACAGAGATGGCGATG G-3', R: 5'-CCACGAGCTCATTCGTAACCTTTGC-3'), *InR* gene (F: 5'-AACAGTGGCGGATTCGGTT-3', R: 5'-TACTCGGAGCATTGGAGGCAT-3'), and for *rp49* control (F: 5'-GACGCTTCAAGGGACAGTATCTG-3', R: 5'-AAACGCGGTTTCAGCATGA-3').

## 2.10 Statistical analysis

Comparisons of two samples were made by either Student's *t*-test or analysis of variance (ANOVA). Graphs were performed using GraphPad Prism 6.0b and Adobe Illustrator software. All statistical comparisons were performed using Microsoft Excel. All data are presented as the mean ± SEM. No asterisk denotes  $P > 0.05$ ; asterisk denotes  $P < 0.05$ ; double-asterisk denotes  $P < 0.01$ ; tripleasterisk denotes  $P < 0.001$ .

## 3 Results and analysis

### 3.1 The distribution of *E. coli* in laboratory-reared and wild-captured fly

Two strains *E. coli* CR0 and *E. coli* CR1 were isolated in laboratory-reared flies with selective agar medium, MAC, on which colonies formed a red or pink colonies. They were further verified to be

facultative anaerobic, gram-negative bacillus, consisting with the features of *E. coli*. The sequencing length of 16S rRNA gene of them showed over 98% identity to *E. coli* (Figure 1). Because two strains isolated from *Drosophila* belonged to *E. coli*, we mainly used the strain of *E. coli* CR0 in this study. Moreover, *E. coli* GZ was isolated from wild-captured flies, suggesting that *E. coli* could be one member of commensals in *Drosophila*. However, *E. coli* CR0 had a relatively distant genetic relationship with the two dominant species, *Lactobacillus plantarum* and *Acetobacter orientalis*, which extended our knowledge of the richness of intestinal microbial flora in *Drosophila*.

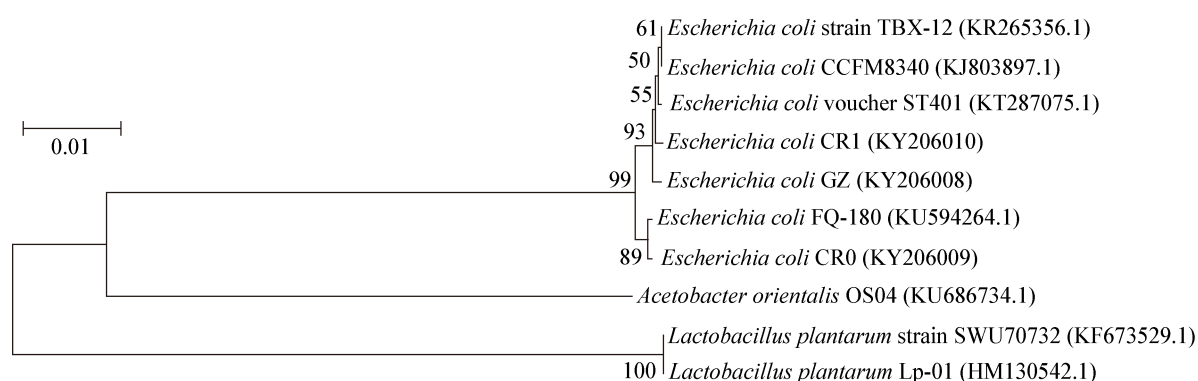
### 3.2 *In vitro* co-existence with commensal bacteria of *Drosophila*

To confirm the indigenous strain of *E. coli* in *Drosophila*, we first assessed the co-existence of *E. coli* with known commensal bacteria *L. plantarum* with *in vitro* fermentation system. As shown in Figure 2A, the OD value of cocultured *E. coli* and *L. plantarum* was higher than any single cultured at 6 h, and slightly lower than single *L. plantarum* but much higher than *E. coli* after 12 h, implicating that they did not compete with each other. Correspondingly, the value of pH in cocultured decreased to the level between *E. coli* and *L. plantarum* following 12 h (Figure 2B). Indeed, the number of cocultured *E. coli* was comparable to *L. plantarum* over time (Figure 2C). For instance, the number of cocultured *E. coli*

was even higher than single *E. coli* at 12 h, and the number of cocultured *L. plantarum* was comparable to single *L. plantarum* at 24 h (Figure 2C), indicating that the two bacteria strains *in vitro* grew well when cocultured. Collectively, our results indicated that *E. coli* was capable of coexisting with commensals of *Drosophila*.

### 3.3 *In vivo* co-existence with commensal bacteria of *Drosophila*

To further confirm the commensal bacterium of *Drosophila*, we then tested the ability of *E. coli* to colonize *Drosophila* gut. GF embryos were cultured on yeast/cornmeal medium supplemented with  $10^8$  CFU of either bacterial species, and internal bacterial loads were quantified at corresponding developmental stages after this inoculation. The data showed that single *E. coli* existed at the whole growth stage of *Drosophila* with the average number of  $10^6$  (Figure 3A), indicating that *E. coli* colonized the whole life cycle of *Drosophila*. The load of *E. coli* in co-cultured group was higher than that in single *E. coli* control at all stages except pupae, suggesting that *L. plantarum* didn't compete the colonization of *E. coli* *in vivo*. In the medium, the number of *E. coli* in co-cultured group was higher than that in single *E. coli* group in the whole growth phase of fly (Figure 3B), suggesting that *E. coli* cocultured with *L. plantarum* even grew better than their single. Taken together, the results suggested that symbiotic *E. coli* of *Drosophila* could co-exist with *L. plantarum*.

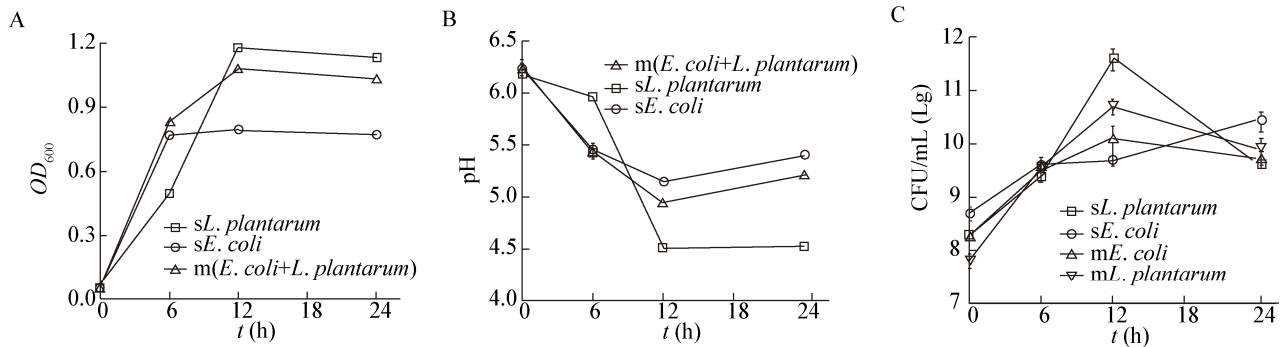


**Figure 1** Phylogenetic tree of *Escherichia coli* and its relatives

图 1 大肠杆菌与相关细菌的系统进化树

Note: Bar: Nucleotide divergence; Number at nodes present bootstrap percentages; Those in parentheses are GenBank accession number.

注: 标尺: 表示序列差异的分支长度; 发育树节点的数值表示 Bootstrap 值; 括号内为 GenBank 数据库的登录号。

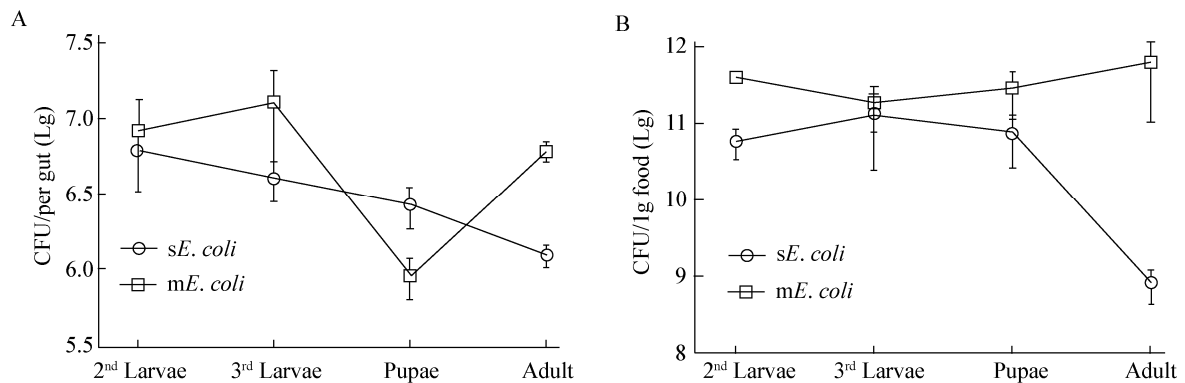


**Figure 2 Co-cultivation of *E. coli* and *L. plantarum* in vitro**

图2 大肠杆菌和乳植杆菌的体外共同培养

Note: A: The curves of growth  $OD_{600}$  value to time course in pure culture and coculture; B: The curves of pH value to time course in pure culture and coculture; C: The curves of CFU value to time course in pure culture and coculture. sL. plantarum: Pure culture of *L. plantarum*; sE. coli: Pure culture of *E. coli*; m (*E. coli*+*L. plantarum*): Coculture of *L. plantarum* and *E. coli*; mL. plantarum: *L. plantarum* in coculture of *L. plantarum* and *E. coli*; mE. coli: *E. coli* in coculture of *L. plantarum* and *E. coli*.

注: A: 纯培养和共培养中生长  $OD$  值对时间曲线图; B: 纯培养和共培养中 pH 值对时间曲线图; C: 纯培养和共培养中菌落形成单位值对时间曲线图. sL. plantarum: 乳植杆菌纯培养; sE. coli: 大肠杆菌纯培养; m (*E. coli*+*L. plantarum*): 乳植杆菌和大肠杆菌共培养; mL. plantarum: 乳植杆菌和大肠杆菌共培养中的乳植杆菌; mE. coli: 乳植杆菌和大肠杆菌共培养中的大肠杆菌.



**Figure 3 Colonization of *E. coli* in fly gut and the medium**

图3 大肠杆菌在果蝇肠道及其培养基中定殖

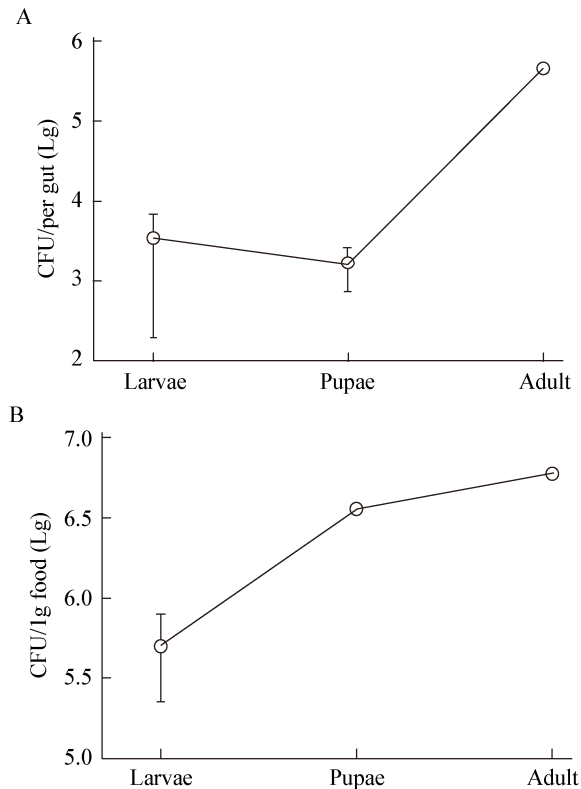
Note: A: The colonization of *E. coli* in *Drosophila* gut. Single *E. coli* and coculture of both *E. coli* and *L. plantarum* were respectively vaccinated to the GF flies, and the number of bacteria per gut was assessed. B: The colonization of *E. coli* in the medium. Single *E. coli* and coculture of both *E. coli* and *L. plantarum* were respectively vaccinated to the GF flies, and the number of bacteria in the medium was assessed.

注: A: 大肠杆菌在果蝇肠道的定殖. 大肠杆菌纯培养液以及大肠杆菌和乳植杆菌共培养液分别接种到 GF 果蝇组, 计数肠道细菌数. B: 大肠杆菌在培养基中的定殖. 大肠杆菌纯培养液以及大肠杆菌和乳植杆菌共培养液分别接种到 GF 果蝇组, 计数培养基中细菌数.

### 3.4 *E. coli* passaged from parental generation to offspring

Since vertical transfer is a hallmark of the natural process of microbiota acquisition, we examined whether *E. coli* could be efficiently transmitted from

parents to their progenies. The results showed that *E. coli* colonized the fly offspring with average CFU rose from  $10^3$  in the stage of larvae and pupae to  $10^5$  in adults (Figure 4A), following the same pattern as the one observed in artificially *E. coli*-associated flies in



**Figure 4** Colonization of *E. coli* in offspring fly gut and the medium

图4 大肠杆菌在子代果蝇肠道及其培养基中定殖

Note: A: The colonization of *E. coli* in offspring *Drosophila* gut; B: The colonization of *E. coli* in the new medium.

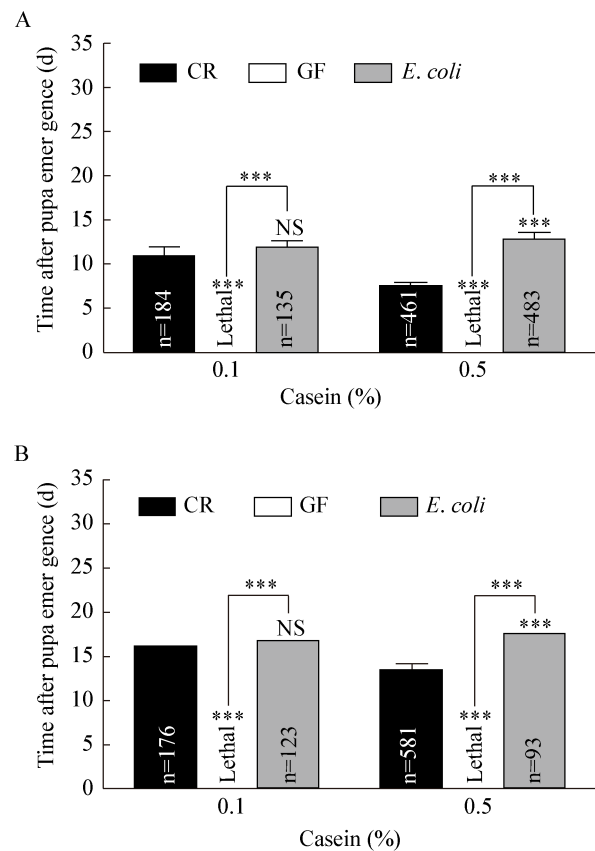
注: A: 大肠杆菌在子代果蝇肠道的定殖; B: 大肠杆菌在新培养基中的定殖.

CR flies. Consistently, the number of *E. coli* in the medium increased from  $10^5$  to  $10^6$  during fly's life cycle (Figure 4B). In conclusion, we demonstrated that *E. coli* transmitted to fly offspring, and that the persistence of *E. coli* was non-fortuitous during the *Drosophila* life cycle.

### 3.5 *E. coli* stimulated the development of *Drosophila*

To gain insight into the potential function of *E. coli*, we sought to evaluate the effects of them on larval/pupal development. The time period spent in each stage is one of the most critical indexes to assay the developmental timing of flies. We artificially removed microbes on the surfaces of eggs and generated GF and gnotobiotic flies as previously described<sup>[15]</sup>. In CR flies with rich nutrition, the average time of development from egg deposition to

pupariation and eclosion was 7 d and 12 d, respectively. This result indicated that rich medium was sufficient to support the development of *Drosophila*. Although CR flies survived in the poor diet with 0.1% casein, the developmental timing of pupal formation and adult eclosion was prolonged to 10.9 d and 16.1 d, respectively. However, none of axenic embryos survived in this autoclaved food beyond the second instar stage even in rich food (Figure 5A), suggesting that *Drosophila*-associated



**Figure 5** *E. coli* stimulated the development of *Drosophila*

图5 大肠杆菌促进果蝇生长发育

Note: A: *E. coli* stimulates the formation of pupae. The timing of pupae formation in three groups (CR, GF and *E. coli*) using the two mediums in a casein-dose manners; B: *E. coli* stimulates the formation of adult. The timing of adult eclosion in three groups (CR, GF and *E. coli*) using the two mediums in a casein-dose manners. \*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ ; \*:  $P < 0.05$ ; NS:  $P > 0.05$ .

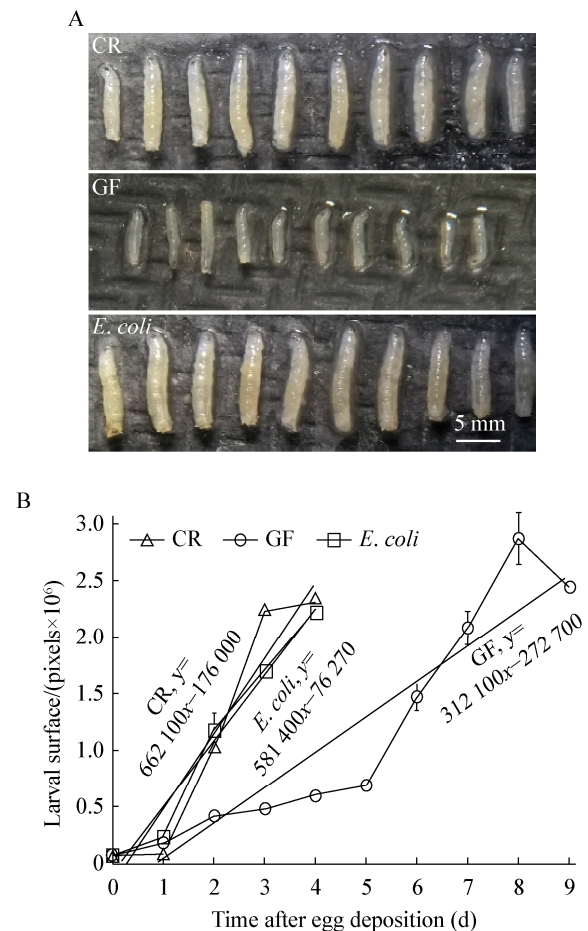
注: A: 大肠杆菌促进蛹形成. 果蝇胚胎在 3 种组别(CR、GF 和 *E. coli*)中成蛹时间, 并使用两种不同酪蛋白浓度的培养基; B: 大肠杆菌促进成虫形成. 果蝇胚胎在 3 种组别(CR、GF 和 *E. coli*)中成虫形成时间, 并使用两种不同酪蛋白浓度的培养基. \*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ ; \*:  $P < 0.05$ ; NS:  $P > 0.05$ .



bacteria were absolutely necessary for the development of hosts. Moreover, we observed that the addition of *E. coli* completely rescued the lethality of GF flies, and partially ameliorated the developmental arrestment of GF flies in fly food. Namely, the average time to puparium and eclosion formation of *E. coli*-inoculated GF embryos in the 0.1% casein medium was 11.9 d and 16.8 d (Figure 5A and B), respectively. The positive effects of *E. coli* on hosts' development were similarly observed in the 0.5% casein medium ( $P < 0.001$ ), albeit of partial rescue. Altogether, our results suggested that *E. coli* recapitulated conventional microbiota to stimulate the development of *Drosophila*.

### 3.6 *E. coli* association promoted larval growth rate

In many metazoans, body size is a key trait that determines the fitness of a species in wild environment, which is subjected to strong evolutionary pressure as well as high adaptative response to environmental conditions. Previous data showed that the length and weight of GF *Drosophila* adult body didn't significantly differ from CR individuals growing on yeast/cornmeal diet<sup>[16]</sup>. Since the developmental timing of GF flies was remarkably prolonged, it was proposed that bacteria could increase the growth rate of larvae. To this end, we examined the growth rate of flies from egg to pupae using the surface area of body. At the beginning (0–1 d), the surface areas of CR and GF flies were comparable, indicating that bacteria was dispensable to promote the growth rate of host at the early stage (Figure 6B). This was partially explained by the fact that most nutrition of embryo and early larvae come from egg yolks. However, the surface area of CR individuals was 3.8-fold more than GF individuals (CR:  $2.3 \times 10^6$  pixels, GF:  $0.6 \times 10^6$  pixels) at day 4 AEL (Figure 6A), suggesting that bacteria accelerated the growth rate of CR larvae at the later stage of larvae. Indeed, the growth rate of CR flies was 2-fold higher than that of GF siblings (Figure 6B). Moreover, the growth rate of *E. coli*-associated flies reminiscent with CR flies, and significantly faster than that in GF ones (Figure 6B,  $P < 0.001$ ), indicating that *E. coli* accelerated the developmental timing of hosts by promoting larval growth rate.



**Figure 6** *E. coli* promoted larval growth rate of *Drosophila*

图6 大肠杆菌提高果蝇幼虫生长速率

Note: A: Body size of representative larvae 96 h AEL under the three conditions (CR, GF, and *E. coli*); B: Larval surface of CR, GF and *E. coli*-associated larvae over time when grown on poor diet (0.5% yeast). Linear regression curves are included (CR,  $y = 662.100x - 176.000$ ; GF,  $y = 312.100x - 272.700$ ; *E. coli*,  $y = 581.400x - 76.270$ ).

注: A: 三组(CR、GF和*E. coli*组)中产卵后96 h幼虫的身体尺寸; B: 低营养食物条件下CR、GF和*E. coli*组幼虫体表面积。计算线性回归曲线(CR,  $y = 662.100x - 176.000$ ; GF,  $y = 312.100x - 272.700$ ; *E. coli*,  $y = 581.400x - 76.270$ ).

### 3.7 *E. coli* promoted Prothoracicotropic hormone and insulin signaling pathway

In *Drosophila*, the onset of the larval-pupal transition is monitored by pulses of the steroid hormone 20-hydroxyecdysone (20HE)<sup>[17]</sup>. Meanwhile, the production and release of 20HE in response to developmental cues is thought to be primarily



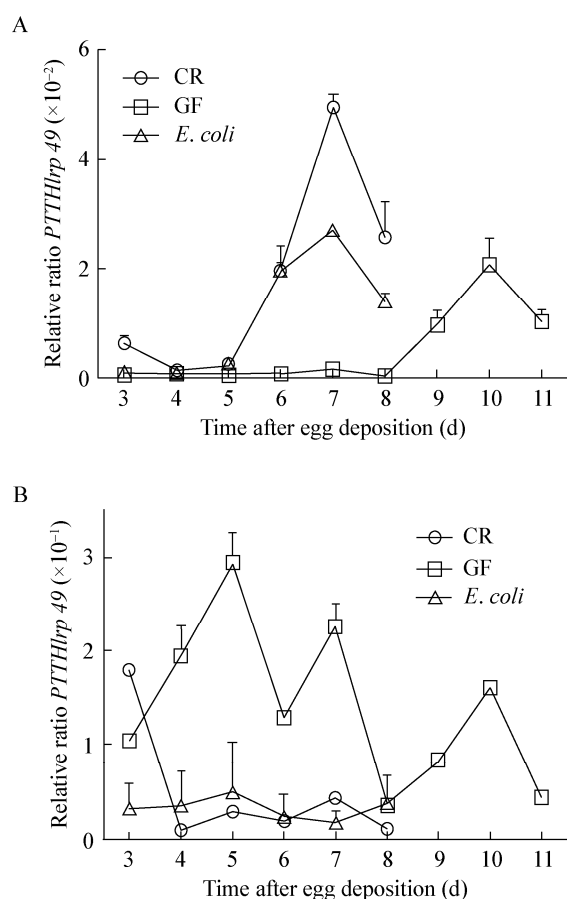
regulated by Prothoracicotropic hormone (*PTTH*)<sup>[18]</sup>. Gene expression of *PTTH* is regulated over time, and reaches the peak in late 3<sup>rd</sup> larva and early pupa period. Thereby, *PTTH* functions as a member of the most important molecular biomarkers for the developing condition in *Drosophila*. RT-qPCR was applied to assay the expression of *PTTH* in time course. As shown in Figure 7A, the peak of *PTTH* expression occurred at the day 7 AEL in CR flies, while the peak was delayed to day 10 AEL in GF counterparts, suggesting that microbiota stimulated the expression of *PTTH*. In addition, control larvae displayed a steep increase in *PTTH* transcription at the end of larval/pupal development, reflecting the surge

of *PTTH* level at that period (Figure 7A). By contrast, the transcription rate of *PTTH* in GF flies only slowly elevated to intermediate levels during the prolonged third larval instar. Likewise, *E. coli* efficiently ameliorated the delayed expression of *PTTH* in GF flies with the peak at day 7 AEL, but the level of *PTTH* expression was still lower comparable to CR flies. The result showed that *E. coli* promoted the secretion of *PTTH* to accelerate the timing of *Drosophila* development.

It was established that commensal bacteria stimulated the development of *Drosophila* via insulin signal pathway<sup>[15]</sup>. InR is a negative molecular marker of the insulin signaling pathway, that is, low InR expression is correlated with high activity of the insulin signaling pathway<sup>[19]</sup>. As shown in Figure 7B, the InR expression of CR larvae was low after day 4 AEL, indicative of activation of insulin signaling pathway. Compared to CR flies, the InR expression of GF flies was higher before day 7 AEL, and mildly declined in later GF larvae, indicating that activity of insulin signal pathway was delayed without commensal bacteria in *Drosophila*. The InR expression was maintained at the low level in *E. coli*-associated larvae, and was comparable to CR larvae (Figure 7B). Taken together, these results showed that *E. coli* participated in activating insulin signal pathway during larval growth.

#### 4 Discussion and Conclusion

In the present study, we revealed that *E. coli* acted as one of the bacterial communities of *Drosophila* and colonized the guts of *Drosophila*. Early studies have showed individual variation within phylotypes occurring in *Drosophila* stocks<sup>[20]</sup>, and our data of symbiotic *E. coli* provide another one symbiotic bacteria in *Drosophila*, enriching the diversity and complexity of microbiotal composition of fly. One study analyzed 11 natural populations of *D. melanogaster* and found that commensal bacteria species richness varied among host locations<sup>[21]</sup>. This result showed that the habitats impacted the abundance and composition of commensal communities. Hence, it was reasonable to isolate *E. coli* (Figure 1) in lab-reared and wild-caught flies. Interestingly, we found that not all wild-captured flies housed *E. coli*. Our result consistently suggested that environmental factors strongly influenced the fly



**Figure 7 Effect of *E. coli* on the expression levels of *PTTH***

图7 大肠杆菌对脑促胸腺激素分泌水平的影响

Note: A: *PTTH* mRNA levels from day 3 AEL to day 11 AEL in CR, GF and *E. coli* groups; B: InR mRNA levels from day 3 AEL to day 11 AEL in CR, GF and *E. coli* groups.

注: A: CR、GF和*E. coli*组产卵后3~11 d体内脑促胸腺激素分泌水平; B: CR、GF和*E. coli*组产卵后3~11 d体内InR水平。

microbiotal composition.

*E. coli* is one of the most diverse microbial species, containing both pathogenic and non-pathogenic strains. Pathogenic *E. coli* can cause diseases, such as urinary tract infections and serious intestinal diseases<sup>[11,22]</sup>. In fact, most *E. coli* are actually part of the normal intestinal microflora which exerts a barrier effect against enteropathogens<sup>[23-24]</sup>. The previous study showed that *E. coli* was considered as one of pathogens in *Drosophila*, because it persisted during the development of the insect only when monoxenic and replaced after exposure to normal flora<sup>[25]</sup>. However, our study revealed that *E. coli* strains were truly symbiotic bacteria in *Drosophila* by the colonization in both parent and progeny (Figures 3 and 4). Moreover, *E. coli* was essential to support the growth and development of *Drosophila* in the corn-casein-glucose meal. Our data suggested that *E. coli* acted as one of symbiotic rather than pathogenic bacteria in *Drosophila* in aspect of developmental timing of hosts. Notably, we recently found that *E. coli* differed from other commensal bacteria, because it induced the egg-laying avoidance of hosts<sup>[26-27]</sup>. Thus, upcoming investigation will further explore the potential traits of distinct indigenous bacteria in *Drosophila* guts.

Insulin signal pathway regulated host homeostatic programs to control developmental rate, body size, energy metabolism, and intestinal stem cell activity<sup>[15]</sup>. It was reported that commensal bacterium, *Acetobacter pomorum*, stimulated host development by modulating insulin/insulin-like growth factor signaling (IIS) in *Drosophila*<sup>[15]</sup>. Our study deciphered that *E. coli* decreased the expression level of InR gene, thus activating insulin signal pathway to promote development of *Drosophila* (Figure 7B), which resembled with previous study. Moreover, the activation of insulin signal pathway correlated with that of *PTTH* to accelerate the larval-pupal transition and *Drosophila* growth rate (Figures 6 and 7). *E. coli*, as a resident microbe in *Drosophila*, might collaborate to digest complex substrates and synthesize nutrition available to assist with the development of *Drosophila*. It makes sense that bacteria thriving on the low casein food surface enriched protein source, and provided fly hosts with a commensal more nutritional food source<sup>[28]</sup>.

In conclusion, our research revealed that *E. coli* was a intestinal flora of *Drosophila* and had important stimulating effects on the developmental timing of *Drosophila*. *E. coli* significantly stimulated the expression of *PTTH* and InR that accelerated the growth rate of hosts. The bacteria-*Drosophila* model could provide a fascinating insight into the relationship of *E. coli* and vertebrates, including human.

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