

## RESEARCH REPORT

**Composition and diversity analysis of intestinal microbiota in the fifth instar silkworm, *Bombyx mori* L.****C Hou<sup>1,2\*</sup>, Y Shi<sup>1</sup>, H Wang<sup>1</sup>, R Li<sup>1</sup>, M A Nartey<sup>1</sup>, X Guo<sup>1,2</sup>**<sup>1</sup>*Jiangsu Key Laboratory of Sericultural Biology and Biotechnology, School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang Jiangsu 212018, China;*<sup>2</sup>*The Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture, Sericultural Research Institute, Chinese Academy of Agricultural Science, Zhenjiang Jiangsu 212018, China*

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**Abstract**

Despite the low diversity and high variability at intra- and interspecific levels, gut microbiota of insect play important roles in digesting and absorbing nutrients, health and development, even in resistance and immunity. In this study, we investigate the composition and diversity of gut microbiota in the 5<sup>th</sup> instar of silkworm larvae since the amount of ingested mulberry leaves of this stage is about 85% of the total amount. Intestinal contents were collected at 0, 12, 24, 48 and 72h in the 5<sup>th</sup> instar. 32, 21, 29, 24, 14 phyla and 386, 163, 292, 196, 144 genera were detected respectively in the samples described above. In general, the dominated phyla in the larvae gut were *Firmicutes*, *Cyanobacteria*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*; the dominated genera were *Enterococcus*, *Staphylococcus*, *Arthrobacter*, *Pseudomonas*, *Lactobacillus*, *Bacteroides*, *Paenibacillus* and *Serratia*, respectively. The range of phyla and genera was different and there were two peaks of microbial diversity at 0 and 24 h. Compared with the intestinal bacteria of these time points, there were unique bacteria and 56 common ones. In these bacteria, the abundance of Gram-positive bacteria including *Enterococcus* and *Staphylococcus* were the highest. Our results provided foundation and reference on the research of intestinal microorganisms and resistance breeding in silkworm.

**Key Words:** Silkworm, Gut microbiota, Composition, Pyrosequencing 16S rRNA genes**Introduction**

The silkworm, *Bombyx mori*, an important economical insects and model organisms of lepidopteran, has contributed enormously to the study of Lepidopteran genetics and immunology (Goldsmith *et al.*, 2005; Hou *et al.*, 2011). The silkworm corporeity is closely related to sericulture production, the related factors of effected corporeity include climatic condition, quality of mulberry leaves, silkworm strains, digestion and absorption of nutrients. Digestion, absorption and disease prevention of silkworm were closely related to the microbiota lived in the silkworm digestive tract (Yuan *et al.*, 2006). Therefore, understanding the change of intestinal bacteria can help us improve the utilization of mulberry and health of the silkworm.

The insects gut inhabits many non-pathogenic microorganisms, which is called a special growth environment of microorganisms. The microbial community structure of insect gut is affected by insect species, the morphology of digestive tract, feed, feeding habits, insect age, pathological conditions and the like (Dillon and Dillon, 2004). These intestinal bacteria play important roles in metabolism, health, reproduction, immunity and pesticide degradation (Maslowski and Mackay, 2011; Simon *et al.*, 2011; Lee and Brey, 2013). They are also involved in host-parasite interaction, thus influence evolutionary and ecological processes of insect by affecting host biology (Feldhaar *et al.*, 2011; Aptedeshpande *et al.*, 2014). Therefore, it is necessary to access the composition and diversity of the intestinal bacteria, which can provide some new insights in development, immunity, new pesticide exploration, even evolution of insect.

Molecular markers had been widely used in analysis of silkworm traits (Li *et al.*, 2007; Xiang *et al.*, 2007; Li *et al.*, 2013). 16S rRNA genes are important references to identify and analyze phylogenetic bacteria, which are common markers

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of bacteria with highly conserved and special sequences (Caporaso *et al.*, 2011). The related study of intestinal microbiota in silkworm was few. Some classical microbiological and molecular techniques had been used to investigate the bacterial composition of the silkworm gut, such as culture-dependent isolation methods and DGGE fingerprinting (Yuan *et al.*, 2006; Xiang *et al.*, 2007). However, these relatively low-throughput techniques cannot provide enough information about the microbial composition and diversity in hosts. Compared with these methods, the recent rapid development of high-throughput sequencing techniques provide new methods in analysis of 16S rRNA genes, genome sequence and transcriptome (Boissière *et al.*, 2012; Hou *et al.*, 2011; 2014; Li *et al.*, 2016; Chengxiang *et al.*, 2017; Zhang *et al.*, 2017). We explored the bacterial compositional changes and diversity at different developmental times by sequencing 16S rRNA genes to understand the host-bacterial relationships in silkworm gut.

## Materials and Methods

### *Silkworm strain*

The silkworm strain Dazao was provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. The silkworm larvae were reared by using fresh mulberry leaves at standard temperature and humidity. The newly exuviated 5<sup>th</sup> instar larvae were used for the experiments.

### *Collection of intestinal contents*

The newly exuviated 5<sup>th</sup> instar larvae were fed with mulberry leaves. The mid guts of 15 larvae were dissected in a sterile environment at five time points (0, 12, 24, 48 and 72 h after feeding with mulberry leaves) respectively. Three repeats of each time points were collected. Prior to dissection, the dissecting apparatus such as scissors and forceps were sterilized by autoclaving and UV treatment. The intestinal contents were immediately collected into the sterile eppendorf tubes, quickly frozen in liquid nitrogen and stored at -80 °C.

### *DNA extraction, PCR amplification, library construction and sequencing*

The genomic DNA of the intestinal contents was extracted using SDS method, then the extracted DNAs were detected by agarose gel electrophoresis and fluorimeter, then the eligible DNA samples were diluted to 1 ng/ul. 16S rDNA Amplification Sequencing was amplified based on Illumina HiSeq sequencing platform and the Paired-End sequencing method, using high-fidelity enzyme and specific primers with Barcode (515F: 5'-GTGCCAGCMGCCGCGGTAA-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso *et al.*, 2011; McHardy *et al.*, 2013). The amplified system was 50 µL contained 10 ng DNA, 5 µL 10x PCR buffer, 0.5 µL 10 mmol/L dNTPs and 5 U/µL Plantium Taq, 1 µmol/L 515F and 806R primer 5 µL, respectively. Thermocycling conditions were: 94 °C for 3 min; 5 cycles of 94 °C for 30 s, 45 °C for 20 s, 65 °C for 30 s; 72 °C for 90 s; then 20 cycles of 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s; finally 72 °C for 5 min. The PCR amplification action used

the Phusion® High-Fidelity PCR Master Mix with GC Buffer kit (New England Biolabs, USA). The amplification product was tested by 2% agarose gel electrophoresis. The aimed amplicons were purified by QIAquick Gel Extraction Kit (qiagen, German) according to the protocols and their concentrations were determined by fluorimeter. The library was constructed by TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) and quantified by Qubit and Quantitative real-time PCR, then the qualified library was sequenced in HiSeq2500 PE250. Among the sequenced sample libraries, there were 3 repeats for each sample of 0, 12, 24, 48, and 72 h in the 5<sup>th</sup> star.

### *Acquisition of effective tags*

Raw tags of samples were obtained from machine data by splitting Barcode sequence and PCR amplification primers, then splicing by Flash (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>). Raw tags must be conformed to the following procedure by strictly filtrating and initially getting the high-quality data (clean tags): 1) tags interception: the first low-quality base site was truncated in raw tags from continuous low quality value (the acquiescent quality threshold was ≤ 19 base) to the setting length (the acquiescent length was 3 base). 2) length filtration of tags: after interception, further filter tags in the continuous high quality base with length was less than 75% of tags length. 3) acquisition of effective tags: the remaining tags after interception and filtration need compare and detected chimera sequences in the gold database ([http://drive5.com/uchime/uchime\\_download.html](http://drive5.com/uchime/uchime_download.html)) and remove them ([http://www.drive5.com/usearch/manual/chimera\\_for\\_mation.html](http://www.drive5.com/usearch/manual/chimera_for_mation.html)), finally obtained the effective tags.

### *Operational taxonomic units (OTUs) clustering, species annotation and diversity analysis*

All the effective tags of samples were clustered into OTUs by uparse software (Uparse v7.0.1001, <http://drive5.com/uparse/>) and the threshold of acquiescent identity was 97%. The representative sequence of OTUs were filtered according to their emerging frequencies and analyzed their species annotation of these sequence by Mothur method and the SSUrRNA database of SILVER (<http://www.arb-silva.de/>), then the information of species annotation (the acquiescent threshold was 0.8-1) and taxonomy were obtained, finally the community composition of sample was counted in each classification level (kingdom, phylum, class, order, family, genus, species). The multiple sequence alignment were confirmed by muscle software (Version 3.8.31, <http://www.drive5.com/muscle/>) and the systematic relationships of OTUs representative sequence were obtained. Finally, all the samples data were treated by homogenization procedure, the treatment was carried out using the least amount in the data as standard, the followed Alpha and Beta diversity analyses, cluster and phylogenetic analysis were based on the normalized data.

Diversity analysis included Alpha and Beta diversity analysis, alpha diversity analysis (<http://scikit-bio.org/docs/latest/generated/skbio.divide>

rsity\_alpha.html) was performed to calculate index of ACE, Chao1, Shannon and Simpson using Qiime software (version 1.7.0), draw rarefaction curves, rank abundance and species accumulation curves by R software (version 2.15.3). ACE and Chao1 were used to calculate the bacterial community richness, while Shannon and Simpson were used to calculate the bacterial community diversity and estimate the total number of species. The higher index value of ACE, Chao1, Shannon and Simpson meant the more community number and diversity of microbiota. The bacterial diversity was evaluated by rarefaction curves, rank abundance and species accumulation curves. Rarefaction curve was a representative value of alpha diversity, it was used to evaluate whether the sequencing was sufficient to cover all taxa or not, which could reflect the abundance of species in the samples. Venn diagrams were drawn with the online Venn tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>), and used to represent their intersecting relations.

Beta diversity analysis was performed to get the maps of the phylogenetic tree, Nonmetric Multidimensional Scaling (NMDS), principal components analysis (PCA) and principal coordinate analysis (PCoA). The UniFrac distance was calculated by Qiime software (version 1.7.0) and analyzed the molecular variance to directly compare the structure differences of bacterial community between two samples (Schloss, 2008), then the phylogenetic tree was constructed by the neighbor-joining method of MEGA6.0. The maps of PCA and PCoA were drawn by R software (version 2.15.3). PCoA was used to compare bacterial structures based on weighted - UniFrac from each library. PCA was utilized to explore the correlation between dominant genera. In the maps of PCA and PCoA, the difference of community structure was determined according to the condition of aggregation and separation between samples. NMDS is commonly used to compare the differences among the samples and evolutionary information. The difference of community composition was small if the distance between two points was close.

### Phylogenetic analysis

Phylogenetic analysis was performed to get the evolutionary and similar relationships. The evolutionary relationships of genera Top100 were acquired by multiple sequence alignment, the relative abundance of these genera was combined and integrated, finally the map of phylogenetic relationships were gained by GraPhlAn software (<http://huttenhower.sph.harvard.edu/GraPhlAn>) (Asnicar *et al.*, 2015). The similar relationships of different samples, Unweighted Pair-group Method with Arithmetic Mean (UPGMA) was used to cluster and analyze samples, construct phylogenetic tree of different samples, finally solve classification problems.

## Results

### Analysis of the sequencing data

After a series of data management, 651,441 effective tags were obtained from the samples by Illumina HiSeq Sequencing platform. 99.43% (647,707) of effective tags was assigned to Taxon and 3,121 OTUs were obtained (Table 1). There were 1200 OTUs in the PC0 group, and 394, 823, 484 and 220 OTUs in PC1, PC2, PC3 and PC4 groups, respectively. Among them, there were 109 shared OTUs (Support Fig 1). After quality controlled, the length of total tags were 18,220,501 bp, length ranging from 191 to 381 bp and the average length was 251.1 bp.

The index values of alpha diversity were calculated by Qiime software (version 1.7.0) (Table 2). In the index values of ACE and Chao1 for calculating bacterial abundance, the values of PC0, PC1, PC2, PC3 and PC4 group were gradually decreased in the index of acachao1 for calculating bacterial abundance, which indicated that their bacterial abundance was also decreasing. This result was consistent with the data in Table 1. In the analysis of alpha diversity, the rarefaction curve showed that bacterial richness in the gut contents was different in 5 samples and they approached to

**Table 1** Sequence data and bacteria in the contents of the healthy silkworm gut

Sample Name	Raw PE	Effective tags	Taxon_Tag	OTUs	Phylum	Class	Order	Family	Genus
PC0	147,772	139,041	137,271	1200	31	58	120	209	368
PC1	150,265	146,121	145,682	394	21	35	78	116	163
PC2	47,301	44,601	43,772	823	29	46	105	169	292
PC3	161,610	156,628	156,070	484	24	43	87	141	196
PC4	168,714	165,050	164,912	220	14	28	55	81	144
total	675,662	651,441	647,707	3,121	119	210	445	716	1,163

The samples of PC0, PC1, PC2, PC3 and PC4 groups were collected from the mid gut contents of five time points in the 5th instar silkworm. The five times points were 0, 12, 24, 48 and 72 h after feed with mulberry leaves

**Table 2** The index values of richness and diversity in the samples

Sample name	Shannon	Simpson	Chao1	ACE	Goods_coverage
PC0	3.771	0.729	1028.908	1021.058	0.995
PC1	1.016	0.241	383.591	417.949	0.997
PC2	5.760	0.844	926.030	898.144	0.997
PC3	1.342	0.428	439.077	469.194	0.998
PC4	0.240	0.039	227.480	250.154	0.999

PC0, PC1, PC2, PC3 and PC4 are samples mentioned in Table 1

the saturation plateau (Fig. 1). This result indicated that sequencing depth has been mainly covered all species in the samples and the bacterial richness of these time points in the silkworm gut could be estimated. It also indicated that species abundance of samples and species abundance of PC0 group was higher than others. The results of rarefaction curve were consistent with the data in Table 1.

Beta diversity analysis was used to measure the changes of species composition on temporal and spatial scales by PCoA, NMDS and PCA. The differences between individuals and/or groups can be observed by PCA and PCoA. NMDS is commonly used to compare the differences among the samples and evolutionary information. The difference of community composition was small if the distance between two points was close.

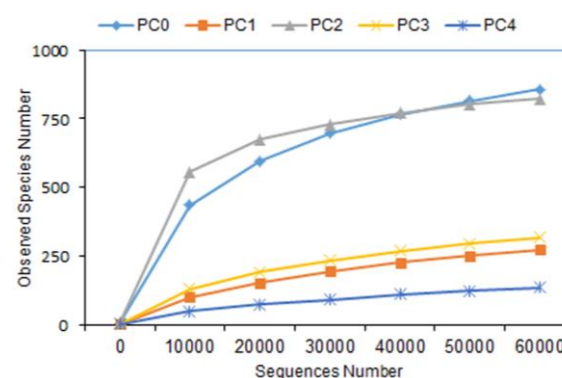
#### Assignment of OTUs and composition of intestinal microbes

A total of 675, 662 Raw PE, 651,441 effective tags and 3121 OTUs were obtained after splitting, splicing and filtering. Eliminating tags representing chloroplast of mulberry leaves, effective tags with 97% identity were clustered into OTUs and these OTUs were assigned to phylum, class, order, family and genus by RDP classifier (Cole *et al.*, 2005). 119 phyla, 210 classes, 445 orders, 716 families and 1,163 genera were detected in the intestinal contents of 5<sup>th</sup> instar silkworm larvae (Table 1). Among of them, the most abundant phyla were Firmicutes (51.41%), Cyanobacteria (32.24%), Actinobacteria (3.07%) and Proteobacteria (6.85%), respectively. The predominant genera were *Enterococcus* (49.11%), *Staphylococcus* (6.66%), *Arthrobacter* (3.45%), *Lactobacillus* (1.04%), *Pseudomonas* (1.07%), respectively.

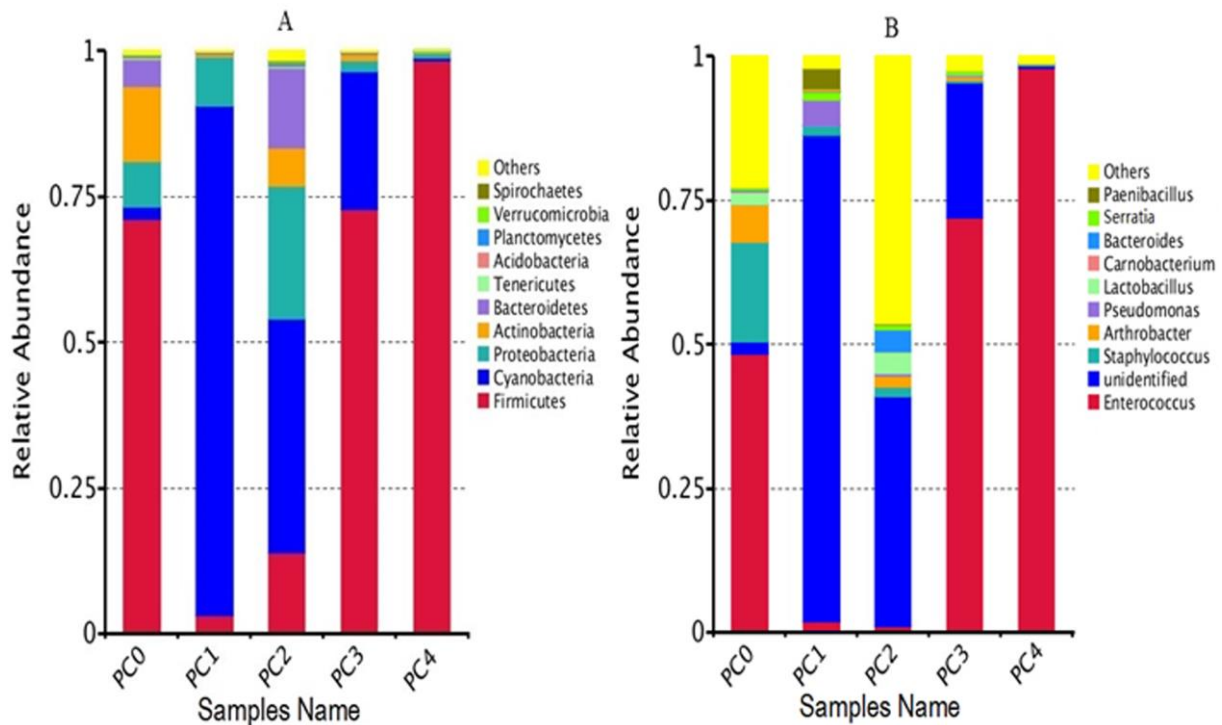
#### Changes of intestinal microbes

To understand the changes of the silkworm intestinal microbes, the changes of composition and abundance of microbes were analyzed at phylum and genus level. The abundance of Firmicutes, Cyanobacteria, Proteobacteria, actinobacteria and Bacteroidetes was significant in mid-gut as shown in the histogram (Fig 2A). In the histogram, the abundance of Firmicutes occupied 71.20%, 3.21%,

13.99%, 72.92%, 98.49% at 0, 12, 24, 48 and 72 h time points, respectively; it decreased during 0-12 h of the 5<sup>th</sup> instar, and increased during 12-72 h. At these five time points, the abundance of Cyanobacteria, Proteobacteria and Actinobacteria was 21.44%, 87.32%, 40.04%, 23.56%, 0.3%, 7.68%, 8.49%, 22.87%, 1.88%, 0.82% and 12.92%, 0.50%, 6.52%, 1.00%, 0.02%, respectively. Among of them, the abundance of Cyanobacteria increased rapidly before 12h, then gradually decreased during 24-72 h; there were no obvious changes about the Proteobacteria abundance before 12 h while Actinobacteria decreased at this time point, then their abundances both increased before 24 h and decreased during 48-72 h.



**Fig. 1** Rarefaction curves of the different samples. PC0, PC1, PC2, PC3 and PC4 are samples mentioned in Table 1. Rarefaction curves of OTUs were clustered according to the acquiescent sequence identity (97%). In this curve, abscissa axis and vertical axis represent the randomly extractive sequences number and OTUs number that can be constructed based on these sequences number, respectively. This curve reflects species abundance and sequencing depth. In this curve, species abundance of PC0 group was higher than others, the quantity of sampling is reasonable for curve tends to be flat



**Fig. 2** The dominant phylum and genera of all the samples at each time point; PC0, PC1, PC2, PC3 and PC4 were samples mentioned in Table 1. The map of A and B were the changes of relative abundance of different bacterial phylum and genera, respectively. Sequences that could not be classified into the known group were assigned as “Unidentified”. In this Fig, the abundance of *Enterococcus* in Firmicutes varied greatly in five samples, furthermore its proportion was higher in PC0, PC3 and PC4 than those of others. The dominant phylum and genera number of PC0 and PC2 was more than other samples

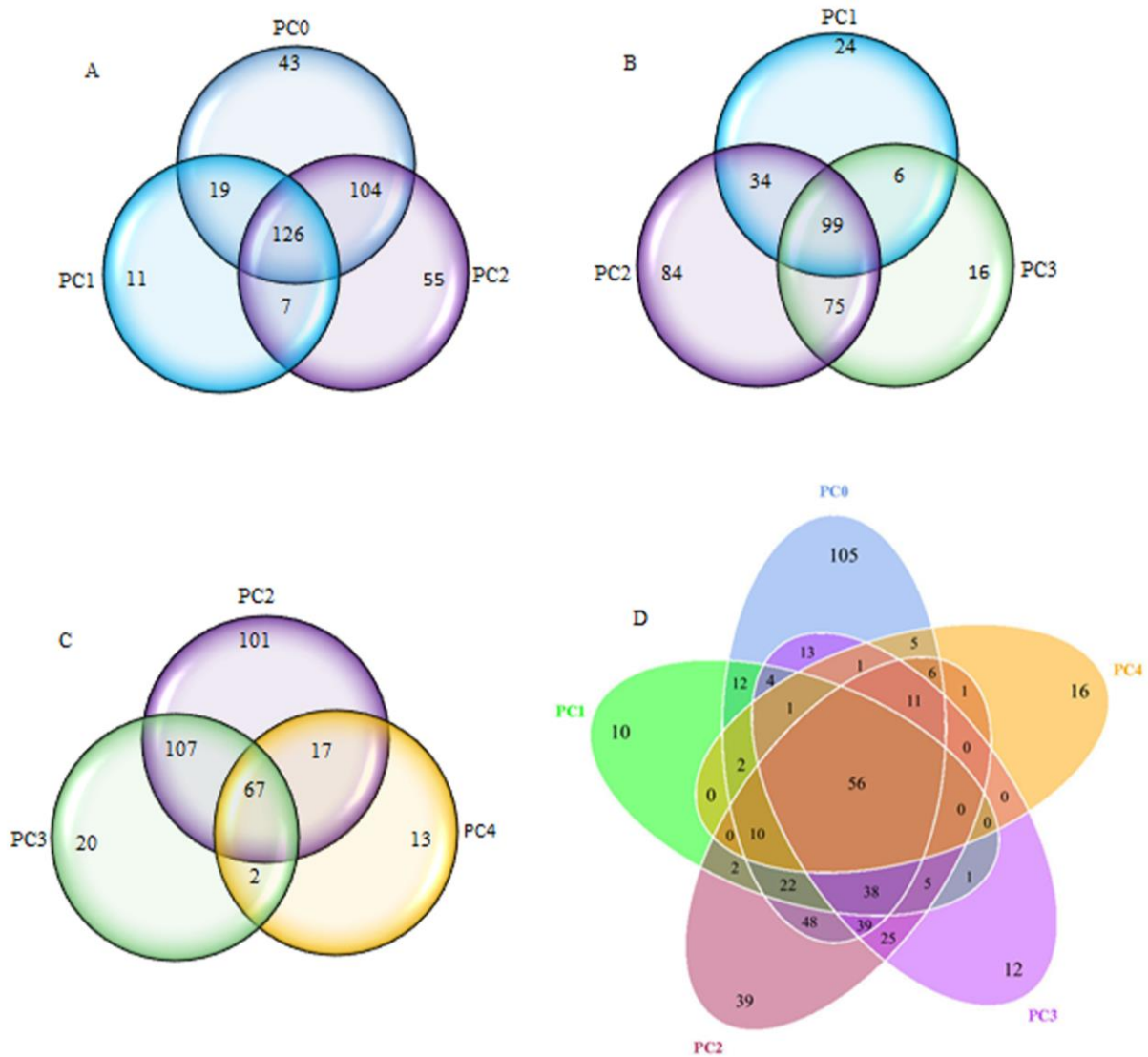
*Enterococcus*, *Staphylococcus*, *Arthrobacter*, *Pseudomonas*, *Lactobacillus*, *Carnobacterium*, *Bacteroides*, *Paenibacillus*, *Serratia*, *Fronidhabitans*, *Brachybacillus* and *Bacillus* were significantly abundant (> 1%). Among of them, *Enterococcus*, *Staphylococcus*, *Lactobacillus*, *Paenibacillus*, *Carnobacterium* and *Bacillus* belong to Firmicutes phylum; *Arthrobacter*, *Fronidhabitans* and *Brachybacillus* are part of Actinobacteria phylum; *Pseudomonas* and *Serratia* pertain to Proteobacteria phylum; *Bacteroides* belongs to Bacteroidetes phylum. The abundance of *Enterococcus*, *Staphylococcus* and *Arthrobacter* at five time points was 48.32%, 1.99%, 0.44%, 71.91%, 98.03%, 17.26%, 1.67%, 1.74%, 0.49%, 0.33% and 6.52%, 0.12%, 1.89%, 0.51%, 0.12%, respectively (Fig 2B). In Fig 2B, the abundance of *Enterococcus* quickly decreased before 24 h, then rapidly increased in the latter time and reached peak at 72 h; the *Staphylococcus* abundance was gradual downward; and the abundance of *Arthrobacter* decreased before 12 h, then increased a little and decreased during 48-72 h.

#### Difference of intestinal microbes at different times

The gut microbes of silkworm were different at five different time points of the 5<sup>th</sup> instar larvae. At phylum level, the number of detected phylum were 31, 21, 29, 24 and 14, respectively. Among them, the

number of phylum with significant abundance (> 1%) were 5, 3, 5, 4 and 1, respectively (Fig. 2A). The abundant changes of Firmicutes, Cyanobacteria, Proteobacteria and Actinobacteria were significant. The abundance of Firmicutes were approximate at 0 h and 48 h, their abundances were 22.18, 5.09, and 0.75 times higher than those of 12 h, 24 h and 72 h, respectively. The abundance of Cyanobacteria increased before 12 h, then gradually decreased. Its abundance of 12 h, 24 h and 48 h was 40.8, 18.68 and 10.9 times higher than that of 0h, while its abundance of 0h was 7.22 times higher than that of 72 h. The abundance of Proteobacteria was basically similar at 0 h and 12 h; compared with the abundance of 0 h, it increased at 24 h and was 3.97 times higher than that of 0 h, then decreased during 48-72 h, and the abundance of 0 h was 2.88 and 6.23 times higher than that of 48 h and 72 h, respectively. The abundance of Actinobacteria was less than the former three phyla, while its abundance changed more rapidly. It decreased before 12 h, increased at 24 h, then continued to decrease until 72 h. Based on the abundance of Actinobacteria at 12 h, the abundance of 0 h, 24 h, 48 h and 72h was 26.37, 13.27, 2.04 and 0.41 times higher than that of 12 h, respectively.

The numbers of detected genera were 368, 163, 292, 196 and 144, and the number of predominant bacterial genera (> 1%) were 8-4-8-2-1 at five time



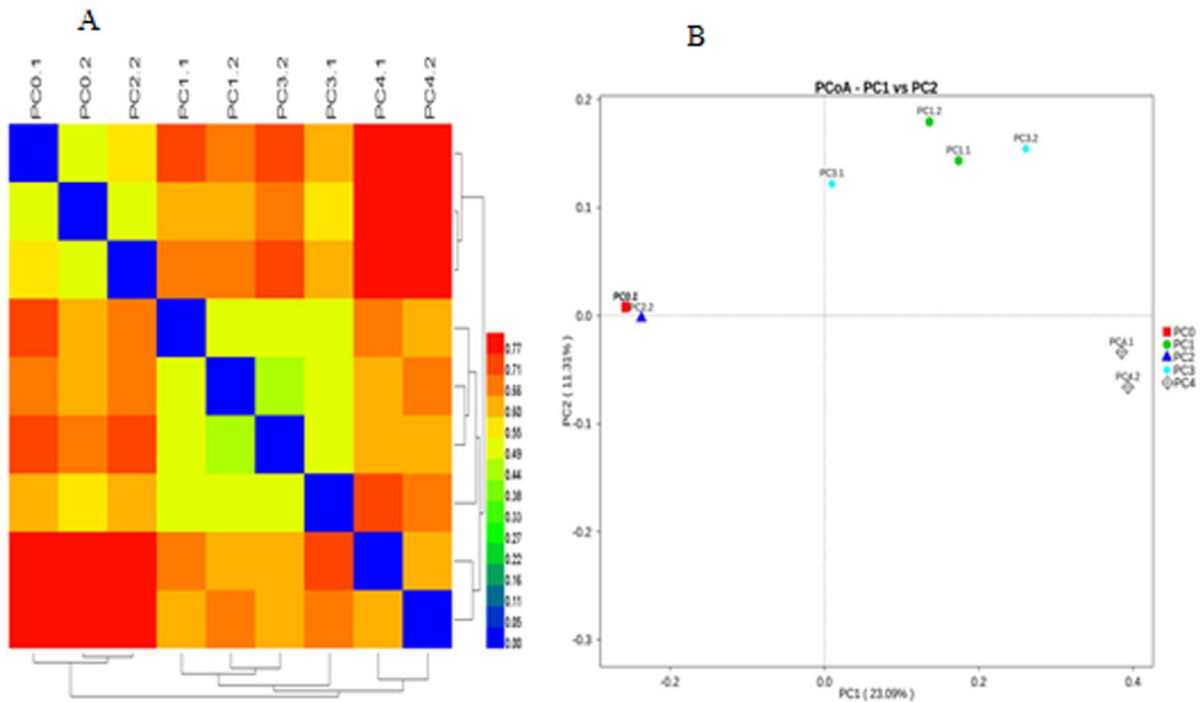
**Fig. 3** Common and unique genera analysis of samples at different time points. The analyses results were showed by Venn diagram; PC0, PC1, PC2, PC3 and PC4 were samples groups mentioned in Table 1. (A) The Venn diagram of PC0, PC1 and PC2 samples groups. (B) The Venn diagram of PC1, PC2 and PC3 samples groups. (C) The Venn diagram of PC2, PC3 and PC4 samples groups. (D) The Venn diagram of all samples groups, including PC0, PC1, PC2, PC3 and PC4 samples groups

points, respectively. Unique genera and common ones exist at the samples of different time points (Fig. 3). Venn diagrams of common and unique genera numbers were constructed to understand genera changes in the 5<sup>th</sup> instar. Compared with detected intestinal microbes among 0 h, 12 h and 24 h, there were 126 common bacteria, 43, 11 and 55 unique ones at each time, respectively (Fig. 3A). There were 99 shared bacteria, 24, 84 and 16 unique ones, among 12 h, 24 h and 48 h (Fig. 3B). 67 common bacteria, 101, 20 and 13 unique ones, were present in the samples of 24 h, 48 h and 72 h, respectively (Fig. 3C). Compared with all detected intestinal microbes at these five time points, there were 56 shared genera, the number of unique ones were 105, 10, 39, 12 and 16, respectively (Fig. 3D).

#### *Cluster analysis of intestinal bacteria in the 5<sup>th</sup> instar silkworm*

According to the phylogenetic relationships between OTUs, the Unifrac distance between samples was calculated by Qiime software (Version 1.7.0), a heatmap displaying the bacterial similarity of different samples by Hemi software and the analytical map of PCoA by R software (Version 2.15.3) were constructed (Fig. 4). All the samples were assigned to three clusters: (I) PC0.1, PC0.2 (two repeats of 0 h in the 5<sup>th</sup> instar) and PC2.2 (the sample of 24 h) were grouped into a cluster. (II) PC1.1, PC1.2 (two repeats of 12 h) and PC3.1, PC3.2 (two repeats of 48 h) were grouped into a cluster. (III) PC4.1 and PC4.2 (two repeats of 12 h) were grouped (Fig. 4A).





**Fig. 4** Heatmap and PCoA map of all samples. UniFrac distance was calculated by Qiime software and used to construct heatmap and PCoA map. (A) Heatmap was constructed by Heml software. Rows and columns represented 5 samples 9 repeats, the similarity represented by the values in the heatmap of microbiota in samples. (B) PCoA map was drawn and analyzed by R software, the distance between two dots represented the similarity of species structure between two samples

In the results map of PCoA, a dot represented a sample. The closer the sample distance, the more similar the species composition structure was. Our PCoA results showed that the samples were clearly separated (Fig. 4B); contribution of the principal components to samples difference, 72.34% and 17.14%, were represented by PC1 and PC2 axis, respectively. PCoA result suggested that the different developmental stage could affect the distribution of the silkworm intestinal bacteria. It showed that the composition of PC0 and PC2 were approached, PC1 and PC3 were nearest. It was also showed that the analysis result of PCoA and heatmap were concordant.

#### *Phylogenetic tree of predominant microbes phylum*

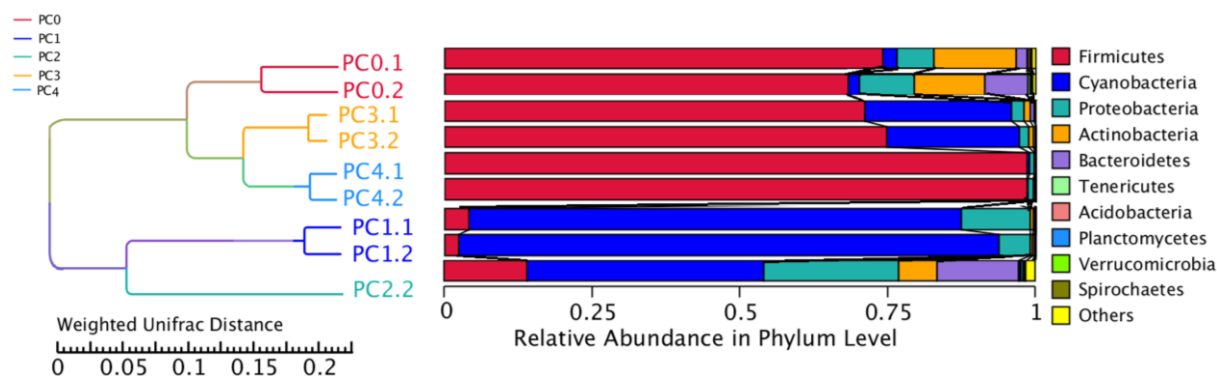
To understand the similarity of different samples, Unweighted Pair-group Method with Arithmetic Mean (UPGMA) was used to cluster and analyze samples, construct phylogenetic tree of different samples, finally solve classification problems. 16S rDNA gene sequences of predominant phylum for PC0, PC1, PC2, PC3 and PC4 were selected to Blast and calculated the UniFrac distance matrix and constructed UPGMA phylogenetic tree (Fig. 5). The components (phylum) of the phylogenetic tree were similar but their abundances were different. Compared the abundance of predominant microbes phylum, PC0, PC3 and PC4 were assigned to one cluster, PC1 and PC2 were assigned to another cluster.

#### *Phylogenetic relationships of genera level*

The relationships of genera Top100 were acquired by multiple sequence alignment, the relative abundance of these genera was combined and integrated, finally the map of phylogenetic relationships were gained by GraPhlAn software (<http://huttenhower.sph.harvard.edu/GraPhlAn>) (Fig. 6). In this map, the evolutionary relationships of genera from different samples, information about the relative abundance and distribution were displayed.

#### **Discussion**

Gut is digestive organ of insect larva, it accounts for about 2/3 of the volume of the body cavity at its maximum in some insect including silkworm. Insect intestinal microbes harbor in digestive tracts with feature of low diversity but high variability at intra- and interspecific levels (Mathieu *et al.*, 2014), they also play important roles in metabolism, development, reproduction, protection, resistance and immunity (Yuan *et al.*, 2006; Xiang *et al.*, 2007; Hedges *et al.*, 2008; Oliver *et al.*, 2009; Vorburger, 2014). In the same insect species, its composition of intestinal microbiota was affected by several factors such as age, genetics and environment, especially diet (Yun *et al.*, 2014; David *et al.*, 2016). Furthermore, the innate immunity and physical barriers of insects limited the diversity of gut microbes by managing bacteria and formation of specific organ to separate the microbes from the host



**Fig. 5** Phylogenetic trees of predominant microbes phylum in different samples. The phylogenetic tree was constructed using MEGA6.0 software and the bootstrap value was 1000 replications. Samples groups PC0, PC1, PC2, PC3 and PC4 were mentioned in Table 1

tissues (Mathieu *et al.*, 2014). The related research about other insect was abundant, but few in silkworm.

The information of microbial compositional variation in the silkworm gut can help us identify the key aspects of microbial composition, and provide references for silkworm breeding. In previous study, two kinds of methods had been used to study intestinal microbes, which were culture-dependent and culture-independent methods. The former was the traditional low-throughput methods, including the methods of isolation and culture-dependent, PCR and 16S rDNA-RFLP; they often provide biased results and the isolated / detected genera were limited (Tian *et al.*, 2007; Xiang *et al.*, 2007). The latter based on the analysis of high-throughput sequencing and open database, they can provide adequate information (Sun *et al.*, 2016). Furthermore, rarefaction analysis could judge whether the sequencing approach was sufficient or not by a plateau and whether the detected bacterial diversity was underestimated.

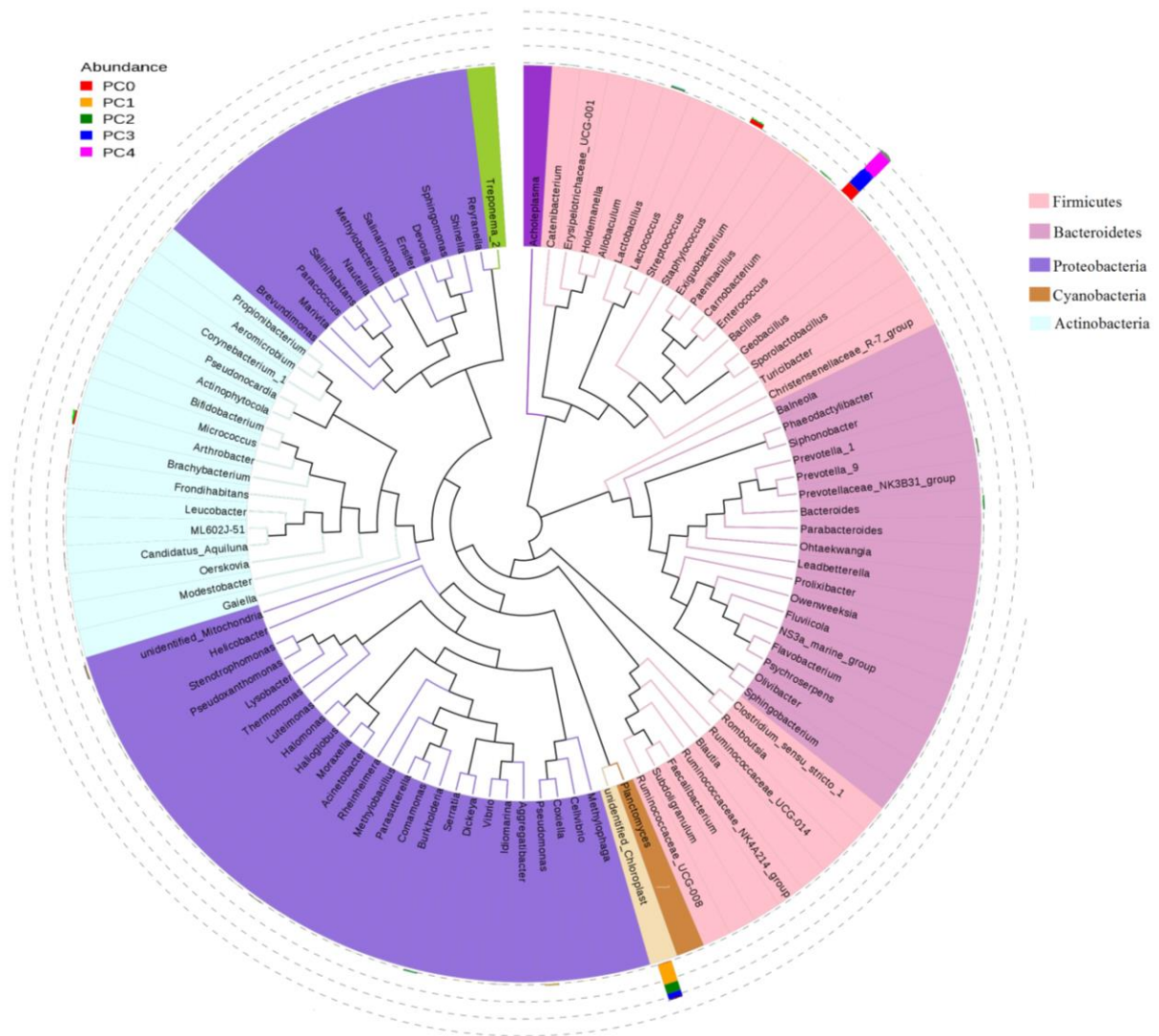
In the identified intestinal microbes of different insects, there were some same phyla in the composition, such as Firmicutes and Proteobacteria; but there were also some differences with the effect of environmental habitat, developmental stage, phylogeny of host, especially diet (Xiang *et al.*, 2010; Cirimotich *et al.*, 2011). There were also some reports about the predominant genera intestinal microbes of silkworm. After the silkworm larvae of Dongting × Bibo strain reared with tricuspid cudrania and mulberry leaves, there were only four shared and dominant genera (*Brevundimonas*, *Stenotrophomonas*, *Enterobacter* and *Staphylococcus*) in their gut (Xiang *et al.*, 2010). In the monophagous C108 and the polyphagous SCN2 strains, the shared predominant phyla and genera were Proteobacteria and Lactobacillales, *Enterococcus* and *Thermus*, respectively (Xiang *et al.*, 2007). In our study some predominant phyla included Firmicutes, Cyanobacteria, Actinobacteria and Proteobacteria, predominant genera included *Arthrobacter*, *Staphylococcus*, *Enterococcus*, *Lactobacillus* and *Pseudomonas* were identified.

Compared with the former research, there were some same phyla and/or genera, such as *Staphylococcus* and *Enterococcus*. Maybe like some polyphagous lepidopteran insect (David *et al.*, 2016), there was a relative stable core microbiota in silkworm. We suspected that silkworm and their core intestinal microbes establish a symbiotic relationship of co-adaptation and co-evolution to maintain survival and reproduction in the evolutionary process.

Some gut microbiotas were related with the organism's resistance, such as *Enterobacter bacterium* (Dillon *et al.*, 2010; Cirimotich *et al.*, 2011). In the intestinal bacteria of silkworm, *Enterococcus* is a high frequency microorganism and can reduce pH of digestive juice and inhibit some pathogens growth in intestinal environment through its products (Takizawa *et al.*, 1968). The functions of *Enterococcus* complement each other. Its antibacterial function is enhanced in the acidic condition, at the same time the pH abatement of intestinal digestive juice can protect hosts against attacks of toxins (Mead *et al.*, 1988; Broderick *et al.*, 2004; Li *et al.*, 2010). The appropriate supplementary of *Enterococcus faecalis* in diets can increase immunity of organisms (Shi *et al.*, 2015). In the prevention and control of silkworm disease, it can be used as a possible probiotic for biological control since its secreta can strongly inhibit the germination of *Nosema bombycis* spores *in vitro* (Lu and Wang, 2002). These research implied that the appropriate supplementary of *Enterococcus in vivo* can enhance immunity of host. In our study, the number of *Enterococcus* increased with the prolongation of feeding period. We suspected that this may be related with resistance and immunity of silkworm.

The 5<sup>th</sup> instar is the key stage of silkworm development, the amount of ingested mulberry leaves of this stage is about 85% of the total amount in the whole larval stage. Intake of mulberry leaves was continuously increased at the 5<sup>th</sup> instar before gluttonous stage, the numbers of detected genera were reduced while the percent of some genera were increased in this study, e.g. *Enterococcus*. It





**Fig. 6** The map of phylogenetic relationships in genera level. Inside circle was a phylogenetic tree of genera level which was constructed by the representative sequences of species. Branch color represented the corresponding phylum (Details see the top right corner of legend). Outside circle of each genus represented the relative abundance in each group, the colors of relative abundance represented different groups. The thermodynamic maps according to the abundance of each genera were then drawn, their values were 10000 times of the original value then converted to the value of log 2

may be related to the resistant and metabolic function of the silkworm, ingestion and digestion substantial mulberry leaves to prepare for the development of silk gland and metamorphosis, meanwhile it also need increase its resistance to defend bad environment and pathogenic microorganism. Furthermore, the last instar is also the critical period for the growth of symbiotic bacteria in host body, the food intake during this period provide the conditions for the growth of commensal bacteria, and this condition is propitious to the common development of insect and its symbiotic bacteria (Mathieu *et al.*, 2014). We thought the resistance and intake of mulberry leaves may be corresponding to the main and/or core microbes of silkworm gut.

Compared with the traditional low-throughput methods, much more information of coverage and diversity of the gut microbes in hosts was detected by high-throughput sequencing techniques (Caporaso *et al.*, 2011; Boissière *et al.*, 2012). 16S rRNAs were used to evaluate the diversity of microbial communities in the gut by high-throughput sequencing techniques, microbial taxonomic groups were determined and compared by OTUs. The changes of proportional OTUs can be observed based on the differences of gut microbe. 16S rRNA gene is not only a highly conserved and special common marker of bacteria, but also can be used to predict the functional microbes in the gut for some diseases which were associated with changes of microbial composition (Turnbaugh *et al.*, 2006; Qin

*et al.*, 2012). Now some corresponding intestinal microbes were used to treat the specific diseases in medicine (Zhang *et al.*, 2013). Microbial symbionts of insects are potential tools which can improve the innate immune homeostasis and contribute to the general insect wellbeing (Crotti *et al.*, 2013).

In this research, we explored the richness and dominance of bacterial in the 5<sup>th</sup> instar silkworm gut by sequencing 16S rRNA techniques, and reported the differential predominance of bacterial strains in five developmental time points. Our results provide some theoretical data about the structure and change of intestinal microbes in 5<sup>th</sup> instar silkworm, which may be used to develop probiotics, increase the conversion rate of leaves and silk, and prevent disease in silkworm in the future. Our further studies about silkworm gut microorganisms will be focused on their composition, acquisition, novel pest control strategies and resistant breeding.

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