Effects of BmCPV Infection on Silkworm <i>Bombyx mori</i> Intestinal Bacteria

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Abstract

The gut microbiota has a crucial role in the growth, development and environmental adaptation in the host insect. The objective of our work was to investigate the microbiota of the healthy silkworm <i>Bombyx mori</i> gut and changes after the infection of <i>B. mori</i> cypovirus (BmCPV). Intestinal contents of the infected and healthy larvae of <i>B. mori</i> of fifth instar were collected at 24, 72 and 144 h post infection with BmCPV. The gut bacteria were analyzed by pyrosequencing of the 16S rRNA gene. 147(135) and 113(103) genera were found in the gut content of the healthy control female (male) larvae and BmCPV-infected female (male) larvae, respectively. In general, the microbial communities in the gut content of healthy larvae were dominated by <i>Enterococcus</i>, <i>Delftia</i>, <i>Pelomonas</i>, <i>Ralstonia</i> and <i>Staphylococcus</i>, however the abundance change of each genus was depended on the developmental stage and gender. Microbial diversity reached minimum at 144 h of fifth instar larvae. The abundance of <i>Enterococcus</i> in the females was substantially lower and the abundance of <i>Delftia</i>, <i>Aurantimonas</i> and <i>Staphylococcus</i> was substantially higher compared to the males. Bacterial diversity in the intestinal contents decreased after post infection with BmCPV, whereas the abundance of both <i>Enterococcus</i> and <i>Staphylococcus</i> which belongs to Gram-positive were increased. Therefore, our findings suggested that observed changes in relative abundance was related to the immune response of silkworm to BmCPV infection. Relevance analysis of plenty of the predominant genera showed the abundance of the <i>Enterococcus</i> genus was in negative correlation with the abundance of the most predominant genera. These results provided insight into the relationship between the gut microbiota and development of the BmCPV-infected silkworm.

Introduction

The silkworm <i>Bombyx mori</i> is an economically important domesticated insect considered as an ideal model organism for Lepidoptera. The yield and quality of cocoons depended on the silkworm strain, climatic condition, silkworm health and absorption of nutrients. Digestive
absorption, nutrient utilization and diseases emergence of the silkworm was closely related to microbiota found in the midgut of the silkworm larvae [1]. Therefore, knowledge of the dynamic change of gut bacteria post infection should lead to the improvement of the health and nutrient absorption of the silkworm.

The dilution culture method of intestinal juice was used to investigate the microbiota in the silkworm gut at different developmental stages, 253 strains of bacteria belonging to 16 genera were isolated[2]. In another study, 89 Enterococcus spp. isolates were detected in the intestinal content of the healthy silkworm larvae and adult moths using the API 20 STREP (V.5.0) system (BIOMERIEUX. A., France) based on numerical taxonomy [3]. Usually, only a few predominant bacterial genera can be isolated by the culture-dependent method; bacterial genera with low abundance tend not to be found with the culture-dependent method. The 16S rRNA gene, common to all prokaryotes, was often used as a marker for identifying bacterial species [4]. Using of the 16S rRNA gene has been a powerful tool for the detection and authentication of bacteria. Further, restriction pattern of the 16S rRNA gene amplified from the gut bacterial metagenomic DNA of silkworm, can be used to detect the bacteria population belonging to the Arthrobacter, Lactobacillus, Pseudomonas, Escherichia, Micrococcus, Bacillus and Staphylococcus genera [1]. Although more additional bacteria can be identified by traditional molecular biology methods compared to the culture-dependent method, diversity and richness of gut microbiota may be underestimated by traditional molecular biology methods.

The silkworm gut microbiota was impacted by forages, four predominant genera (Brevundimonas, Stenotrophomonas, Enterobacter and Staphylococcus) were shared by silkworms cultivated on leaves of Cudrania tricuspidata or mulberry (Morus spp.). Five additional abundant genera (Aeromonas, Brevibacterium, Citrobacter, Escherichia and Klebsiella) and two additional abundant genera (Pseudomonas and Agrobacterium) were recorded from the gut microbiota of silkworm larvae cultivated on mulberry and C. tricuspidata leaves respectively [5]. The distribution of intestinal bacteria was also changed with regard to the state of health of the silkworm; the number of bacterial species of the genus Enterococci decreased, while the number of bacteria of the genus Enterococci was increased in silkworms infected with Nosema bombycis which caused the epidemic disease pebrine compared to healthy silkworms [6].

Bacterial intestinal diseases of B. mori can be caused by abnormal multiplication of bacteria in the gut; B. mori cypovirus (BmCPV) specially infected the epithelial cells of the silkworm midgut, silkworm cytoplasmic polyhedrosis caused by infection with BmCPV usually accompanies silkworm bacterial intestinal disease. To understand the effect of BmCPV infection on silkworm gut microbiota, in the present study, we explored the difference of microbiota between healthy female and male silkworm larvae and the changes of bacterial diversity after infection with BmCPV using pyrosequencing of the 16S rRNA gene. The results indicated that 147 and 135 genera were detected in the gut of healthy female and male silkworm larvae, respectively. The diversity of bacterial microbiota was reduced post infection with BmCPV; only 113 and 103 genera were observed in the female and male silkworm larvae, respectively. These results provided insight into the relationship between the gut microbiota and development of the BmCPV-infected silkworm.

Materials and Methods
Collection of intestinal contents
B. mori larvae of Daizo strain were cultured at 25°C on mulberry leaves and at a suitable humidity of about 70% ±5% together with a photoperiod of 14 h of light and 10 h of dark. Polyhedra were extracted from silkworm intestine which have infected with BmCPV, it has purified by multi-layer gauze and centrifuged using 6000r/min and then resuspended in 1×PBS to 10^8 polyhedra ml^-1.
The newly molted fifth instar larvae were fed for 8 h on leaves smeared with BmCPV (10^6 polyhedra ml^-1) and then fed on untreated leaves. The midgut of 30 larvae (15 females and 15 males) was dissected out in a sterile environment at 24, 72 and 144 h post infection, respectively. The collected intestinal contents were immediately frozen and stored at –80°C. Silkworms fed for 8 h on mulberry leaves smeared with sterilized double distilled water were used as control.

DNA extraction, amplification, purification and sequencing

Total genomic DNA was extracted from the intestinal contents using the Z.E.N.A Soil DNA Kit (Omega Bio-Tek, GA, D5625-01). The quality of the extracted DNA was assessed by electrophoresis in 1% (w/v) agarose gel. The concentration of the extracted DNAs was determined using a Qubit® 2.0 Fluorometer (Life Technologies, California USA) and then normalized to 10 ng μl^-1.

Universal 16S rRNA genes were amplified by PCR in a volume of 50 μl containing 10 ng DNA, 5 μl 10 × PCR buffer, 0.5 μl each dNTP (10 mM), 0.5 μl Plantium Taq (5 U μl^-1), 0.5 μl bar-primers (50 μM) V1F:(5ʹ-CGTATCGCCCTCCCTCGGCCATCAG(barcode)AGAGTTTGATCMTGCTCAG-3ʹ) and V3R2: (5ʹ-CTATGCGCCTTGCCAGCCCGCTAGGTATTACCGCGGCTGCTGGCAG-3ʹ) modified at the 5’ end to contain the 454 FLX Titanium Lib L adapters B (italics and underlined) and A (italics and underlined), respectively. The forward primers also contained a six base barcode sequence located between the primer sequence and the adapter. A unique barcode was used for each sample [7]. The thermocycling protocol was as follows: 94°C for 3 min, then 5 cycles of 94°C for 30 s, 45°C for 20 s, 65°C for 30 s, followed by 20 cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. Amplicons were purified using PCR purification kit (Sangon, Shanghai, China), and quantified by Qubit® 2.0 fluorometer (Life Technologies, California, USA) and then pooled for 454 pyrosequencing by the Encode Genomics Bio-Technology Co., Ltd., Suzhou, China.

Analysis of sequence data

Initially, the SFF file output from the sequencer was converted into fasta and qual files using the sffinfo program included in the 454 Life Sciences software package (Roche Diagnostics, Basel). Samples were distinguished by the barcode sequences and de-multiplexed reads were processed using LUCY (version 1.2) [8] to filter out reads with low-quality segments. Each valid read was had the following criteria; (1) contain a primer sequence ≥50 bp long; (2) contain no ambiguous base; (3) match the primer; and (4) be one of the used barcode sequences. Unique sequences were clustered into operational taxonomic units (OTUs) defined at the 97% similarity threshold. Taxonomical classification of the OTU-representative reads down to the genus level was carried out using Mothur’s version of the Ribosomal Database Project (RDP) Bayesian classifier through a normalized RDP training dataset [9]. The relative abundances of individual OTUs in a given assembly were estimated as the percentage of each individual OTU DNA relative to the sum of the total amplified DNA. Alpha diversity analysis was performed by Mothur software [10]. Rarefaction curves were used to assess species richness [11]. The Shannon–Wiener and Simpson diversity indexes were adopted to evaluate the bacterial diversity [12]. Chao1 [13] and Ace [14] indexes were used to estimate the total number of species in samples. The OTUs were aligned through PyNAST with a minimum alignment length of 150 bp and a minimum identity of 75% [15]. After alignment, PH LANE mask (http://greengenes.lbl.gov/) was used to screen away the hypervariable regions.

Relevance analysis between samples

The phylogenetic tree was inferred using the neighbor-joining method with MEGA6.0 [16, 17]. Principal coordinate analyses (PCoA) were carried out using R software version 2.10.0/R
Development Core Team, 2009, http://www.r-project.org) to compare bacterial community structures based on weighted-UniFrac from each library. The weighted-UniFrac distances were subjected to analysis of molecular variance (AMOVA) in Mothur to compare significant differences between bacterial communities from each sample [18, 10]. Principal components analysis (PCA) with linear ordination methods was utilized to explore the correlation between dominant genera using CANOCO 4.5 according to ter Braakand Šmilauer [13]. A histogram was created using SPSS19.0 version on the basis of genera distributed among samples [14]. Venn diagram curves were created with the online tool Venny (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Results

Analysis of the pyrosequencing-derived dataset

After removal of low-quality reads, 60,551 valid reads were obtained from 12 samples using 454 pyrosequencing of the 16S rRNA gene. The total reads were 61,296,879 bp long, each ranging from 50 to 1150 bp with average of 468.9 bp. The number of reads differed for different samples; the number of reads for the 12 samples ranged from 1075 to 13,312. A total of 71.13% (43,072) of the total valid reads was assigned to a genus and 21,020 OTUs were obtained (Table 1). The richness/rarefaction curves for individual samples showed bacterial richness in the gut contents was different among 12 samples, although the rarefaction curves did not tend to approach the saturation plateau (Fig 1A), indicating the true bacterial richness in the silkworm gut was underestimated. Bacterial diversity was estimated by the Shannon index; the curves tended to plateau (Fig 1B) and the Shannon index of the gut microbiota of healthy silkworms was higher compared to BmCPV-infected silkworms, showing bacterial species diversity in the healthy silkworm gut was greater when compared with the BmCPV-infected silkworm gut and a similar result was obtained by Simpson index analysis. The OTU number of a bacterial community was estimated by Chao1 and Ace, the results indicated that

| Samples  | valid reads | Reads assigned to genus | Phylum | Class | Order | Family | Genus | OTUs |
|----------|-------------|-------------------------|--------|-------|-------|--------|-------|------|
| CK-24-M  | 4377        | 2308                    | 11     | 17    | 28    | 55     | 95    | 2479 |
| CK-24-F  | 7084        | 5117                    | 10     | 14    | 26    | 57     | 93    | 4111 |
| CK-72-M  | 2880        | 1395                    | 8      | 13    | 22    | 52     | 83    | 1532 |
| CK-72-F  | 3226        | 1365                    | 12     | 15    | 26    | 55     | 81    | 1637 |
| CK-144-M | 8979        | 7696                    | 8      | 9     | 16    | 29     | 41    | 5116 |
| CK-144-F | 1872        | 922                     | 5      | 11    | 18    | 39     | 70    | 1036 |
| Total CK | 28418       | 18803                   | 16     | 29    | 40    | 94     | 199   | 12335|
| CPV-24-M | 5511        | 3218                    | 11     | 17    | 23    | 54     | 91    | 2398 |
| CPV-24-F | 2157        | 791                     | 10     | 19    | 26    | 51     | 74    | 1363 |
| CPV-72-M | 1075        | 303                     | 6      | 9     | 17    | 28     | 39    | 863  |
| CPV-72-F | 2146        | 866                     | 8      | 12    | 22    | 43     | 72    | 1361 |
| CPV-144-M| 7932        | 7011                    | 3      | 3     | 5     | 6      | 7     | 4400 |
| CPV-144-F| 13312       | 12080                   | 5      | 18    | 12    | 19     | 28    | 5564 |
| Total-CPV| 32133       | 24269                   | 14     | 26    | 35    | 77     | 156   | 12764|

CK-24-F (M), CK-72-F (M) and CK-144-F (M) samples collected from the midgut contents of the fifth instar female (male) silkworm at 24, 72 and 144 h post feed with leaves smeared with water

CPV-24-F (M), CPV-72-F (M) and CPV-144-F (M) samples collected from midgut contents of the fifth instar female (male) silkworm at 24, 72 and 144 h post infection with BmCPV, the data for Chloroplast are omitted.

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community richness of silkworm gut depended on the developmental stage, gender and state of health, community richness of BmCPV-infected silkworm was greater compared to healthy silkworm (Table 2).

### Composition of gut microbiota of the healthy silkworm

A total of 28,418 valid reads and 12335 OTUs were obtained after filtering out reads with low-quality segments. Because there are many chloroplast of mulberry in the intestinal contents of silkworm, the OTU-representative reads were assigned to phylum, class, order, family and genus using the RDP classifier [19] except reads representing chloroplast; 16 phyla, 29 classes, 40 orders, 94 families and 199 genera were detected in the intestinal contents of healthy fifth instar silkworm larvae (Table 1). Where 16 phyla namely Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Armatimonadetes, TM7, Thermotogae, Acidobacteria, OP11, Nitrosira, Gemmatimonadetes, Planctomycetes, Chloroflexi, Deinococcus-Thermus, Verrucomicrobia and Chlorobi were recorded, of which three most abundant phyla were Firmicutes (58.92%), Proteobacteria (39.41%) and Actinobacteria (1.28%).
The predominant genera in the gut contents of healthy silkworm larvae were determined to understand the other important bacteria. The predominant genera (>1%) were sequences related to *Enterococcus* (37.40%), *Delftia* (8.35%), *Pelomonas* (3.38%), *Ralstonia* (2.42%), *Tepidimonas* (1.97%), *Aurantimonas* (1.86%), *Pseudomonas* (1.63%), *Aspromonas* (1.56%) and *Staphylococcus* (1.06%).

Changes of intestinal microbiota of the healthy silkworm during the growth period

To understand the effect of developmental stages of silkworm on the intestinal microbiota, the changes of composition and abundance of bacteria in gut contents of healthy silkworm were determined according to the OTU-representative reads. At phylum level, the abundance of Proteobacteria and Actinobacteria decreased during the growth period of the fifth instar, especially from 72 to 144 h; other bacteria phyla accounted for smaller percentages in samples and displayed no regular pattern of change in fifth instar larvae. At genus level, differences in the composition of the bacterial community in the gut were also found at different developmental stages in the fifth instar. The proportions of some genera at different time points are given in S1 Table. Overall, the proportions of *Pelomonas*, *Ralstonia*, *Tepidimonas*, *Pseudomonas*, *Aspromonas*, *Staphylococcus* and *Aquabacterium* decreased with development of the silkworm. The abundance values of *Methylbacterium*, *Acinetobacter*, *Undibacterium* and *Propionibacterium* at 24 h of the fifth instar larvae were similar to those at 72 h and decreased at 144 h. The abundance of *Enterococcus* was the lowest at 72 h of the fifth instar larvae and the highest at 144 h. The abundance of *Delftia* increased with maturity, reached highest at 72 h of the fifth instar larvae and decreased at 144 h (Fig 2).

Difference in the gut microbiota of the healthy silkworm between the male and female

At phylum level, the abundance of Actinobacteria in male larvae were substantially similar to female larvae at both 24 and 72 h of the fifth instar, whereas the abundance was decreased in

| Samples   | Chao1     | Shannon | Ace     | Simpson |
|-----------|-----------|---------|---------|---------|
| CK-24-F   | 1119.85   | 5.74    | 1187.01 | 0.91    |
| CK-72-F   | 1223.89   | 2.82    | 1399.22 | 0.45    |
| CK-144-F  | 745.64    | 5.68    | 879.45  | 0.91    |
| CK-24-M   | 1085.98   | 6.05    | 1257.32 | 0.89    |
| CK-72-M   | 1328.16   | 3.38    | 1441.29 | 0.55    |
| CK-144-M  | 644.31    | 3.60    | 757.17  | 0.73    |
| CPV-24-F  | 1207.82   | 1.92    | 1394.65 | 0.29    |
| CPV-72-F  | 1251.55   | 2.45    | 1436.79 | 0.38    |
| CPV-144-F | 255.59    | 1.69    | 370.33  | 0.37    |
| CPV-24-M  | 1193.34   | 3.05    | 1311.04 | 0.47    |
| CPV-72-M  | 1053.20   | 1.58    | 1157.05 | 0.24    |
| CPV-144-M | 164.0     | 1.69    | 165.70  | 0.46    |

CK (CPV)-24(72,144)-F (M) are samples mentioned in Table 1. Chao1 and ACE indexes estimated the total number of species in samples. The Shannon–Wiener diversity index was used to assess bacterial diversity and its value positive correlation with microbial diversity. Simpson index was used to quantify the biodiversity of a habitat, its value negative correlation with microbial diversity.

The predominant genera in the gut contents of healthy silkworm larvae were determined to understand the other important bacteria. The predominant genera (>1%) were sequences related to *Enterococcus* (37.40%), *Delftia* (8.35%), *Pelomonas* (3.38%), *Ralstonia* (2.42%), *Tepidimonas* (1.97%), *Aurantimonas* (1.86%), *Pseudomonas* (1.63%), *Aspromonas* (1.56%) and *Staphylococcus* (1.06%).

Changes of intestinal microbiota of the healthy silkworm during the growth period

To understand the effect of developmental stages of silkworm on the intestinal microbiota, the changes of composition and abundance of bacteria in gut contents of healthy silkworm were determined according to the OTU-representative reads. At phylum level, the abundance of Proteobacteria and Actinobacteria decreased during the growth period of the fifth instar, especially from 72 to 144 h; other bacteria phyla accounted for smaller percentages in samples and displayed no regular pattern of change in fifth instar larvae. At genus level, differences in the composition of the bacterial community in the gut were also found at different developmental stages in the fifth instar. The proportions of some genera at different time points are given in S1 Table. Overall, the proportions of *Pelomonas*, *Ralstonia*, *Tepidimonas*, *Pseudomonas*, *Aspromonas*, *Staphylococcus* and *Aquabacterium* decreased with development of the silkworm. The abundance values of *Methylbacterium*, *Acinetobacter*, *Undibacterium* and *Propionibacterium* at 24 h of the fifth instar larvae were similar to those at 72 h and decreased at 144 h. The abundance of *Enterococcus* was the lowest at 72 h of the fifth instar larvae and the highest at 144 h. The abundance of *Delftia* increased with maturity, reached highest at 72 h of the fifth instar larvae and decreased at 144 h (Fig 2).

Difference in the gut microbiota of the healthy silkworm between the male and female

At phylum level, the abundance of Actinobacteria in male larvae were substantially similar to female larvae at both 24 and 72 h of the fifth instar, whereas the abundance was decreased in
both male and female larvae, and the abundance in females was 4.24 times higher than in males. Obvious difference in the richness of Proteobacteria was not found between male and female larvae either at 24 or 72 h of the fifth instar. The abundance in females was 3.17 folds higher than in males. The abundance of Firmicutes decreased in female larvae during development but increased in male larvae.

The gut microbiota in the larvae contained 147 genera in females and 135 in males, 64 genera were found only in females and 52 found only in males; 83 genera were recorded in both genders (Fig 3). The abundance of predominant bacterial genera in the female (male) larvae was: Enterococcus, 24.75% (46.89%); Delftia, 12.57% (5.19%); Pelomonas, 3.27% (3.46%); Ralstonia, 2.21% (2.58%); Tepidimonas, 2.13% (1.85%); Aspromonas, 1.39% (1.69%); Pseudomonas, 1.61% (1.64%); Aurantimonas, 2.75% (1.64%); Acinetobacter, 0.68% (0.44%); and Methylobacterium, 0.48% (0.43%). In general, the abundance of Enterococcus in females was substantially lower when compared with males, and the abundance of Delftia, Aurantimonas and Staphylococcus were substantially higher.

Change of intestinal microbiota after infection with BmCPV

BmCPV specially infects the epithelial cells of the silkworm midgut, and as the disease progresses, white wrinkles typically occurred in the posterior part of the midgut, and consequently, the digestive and absorptive functions of the midgut severely affected. To estimate the effect of
BmCPV infection on gut microbiota, the change of gut microbiota after infection with the BmCPV was investigated. There were marked differences in the composition of the intestinal microbiota in BmCPV-infected silkworms compared to control healthy silkworms. 14 phyla, 26 classes, 35 orders, 77 families and 156 genera of bacteria were detected in the intestinal contents of BmCPV-infected silkworms, fewer compared to the healthy silkworm at all levels of classification (Table 1), suggesting bacterial diversity in the intestinal contents decreased post infection with BmCPV. The three most abundant phyla in BmCPV-infected larvae were Firmicutes (85.76%), Proteobacteria (13.13%) and Actinobacteria (0.91%) recorded, and the abundance of Firmicutes was increased by 45.55%, whereas abundances of Proteobacteria and Actinobacteria were respectively decreased by 66.68% and 28.91% compared to the healthy silkworm.

In genus level, the predominant (>1%) genera post infection were Enterococcus (59.18%), Staphylococcus (4.58%), Delftia (2.70%), Pseudomonas (2.34%) and Methylobacterium (1.28%), roughly, the abundances of Enterococcus and Staphylococcus were increased and the abundance of Delftia was decreased after infection with the BmCPV.

The proportion of bacteria in the intestinal contents changed with the time course of BmCPV infection and the pattern of change depended on the type of bacteria present. The proportion of Enterococcus spp. decreased substantially at 24 h post infection with BmCPV and then increased to 88.75% at 144 h, which was a 1.28-fold increase compared to the control. The abundance of Pseudomonas spp. at 24 h post infection was 2.47-fold higher compared to the control and then decreased with the time course of BmCPV infection, reaching 0.02% at 144 h, which was 27-fold lower compared to the control. The abundance of Staphylococcus spp. at 24 h post infection was 17.16%, which was 9.28-fold greater than control, and then decreased to
0.37% at 144 h, which was similar to the control. The abundance of *Delftia* spp. decreased with the time course of BmCPV infection; the abundance at 24, 72 and 144 h post infection was lower compared to the control (S1 Table).

The change of bacterial microbiota in the BmCPV-infected female silkworms were different compared to the male; 113 genera were found in the females and 103 in the males, 53 unique genera were found in the females and 43 in the males, and 60 genera were found in both genders. The diversity of intestinal bacterial microbiota in BmCPV-infected female and male silk-worm larvae decreased compared to the control. At 144 h post infection, the number of genera detected in the gut contents decreased sharply; only 28 genera were detected in females and 7 in males, compared to 70 and 41, respectively of control (Fig 2).

Post infection with BmCPV, roughly, the abundance of *Delftia*, *Pelomonas*, *Ralstonia*, *Tepidimonas*, *Aspromonas* and *Aurantimonas* genera decreased in both genders. The abundance of both *Enterococcus* and *Staphylococcus* increased in females but there was no significant change in males. Post infection with BmCPV, the richness of *Pseudomonas* was decreased in females and increased in males. Whereas, the abundance of *Acinetobacter* and *Methylobacterium* genera did not showed any change in females post infection with BmCPV; however, the abundance of *Acinetobacter* decreased and *Methylobacterium* increased in males (Fig 2).

**Similarity of bacterial communities in the midgut of BmCPV-infected and healthy control silkworms**

A samples distance matrix was calculated by Unifrac software and a heatmap displaying the similarity of bacterial communities in different samples were generated (Fig 3A). Each sample was assigned to one of three clusters: (1) CK-24-M, CK-24-F (the bacterial community of the gut contents at 24 h in the fifth instar male and female larvae, respectively) and CK-144-F (the bacterial community of the gut contents at 144 h in fifth instar female larvae) were grouped into a cluster. (2) CPV-144-F and CPV-144-M (the bacterial community of the gut contents at 144 h of the fifth instar female and male larvae, respectively, post infection with BmCPV) and CK-144-M (the bacterial community of the gut contents at 144 h in fifth instar male larvae) were grouped into a cluster. (3) Other samples were grouped into a cluster, indicating samples were not clustered completely according to sample type (Fig 3A). A similar result was obtained by principal coordinate analysis (PCoA), the samples were clearly separated in the PCoA plot, 51.74%, 33.34% and 6.53% of total variation could be explained by the PC1, PC2 and PC3 axis, respectively (Fig 3B), suggesting the intestinal bacteria community could be affected by gender, development and infection with BmCPV.

Further to understand changes of bacterial microbiota with gender, developmental stages and the time course of BmCPV infection, Venn diagrams were constructed. Altogether, 35 and 29 genera were found in all samples of the gut contents of healthy female and male larvae, respectively, at different developmental stages of the fifth instar (Fig 4A and 4B) and 24 genera were detected in all larvae (Fig 4E). Post infection with BmCPV, the number of shared genera was reduced; 16 and 6 genera were present in all samples from infected females and males, respectively, (Fig 4C and 4D) and only 6 genera were shared by the female and male larvae (Fig 4E). 15 genera were shared by females and 5 covered by males before and after infection, and only 5 genera (*Enterococcus, Delftia, Pelomonas, Staphylococcus* and *Petrobacter*) were shared by all infected larvae (Fig 4E).

**Phylogenetic tree of predominant genera**

To understand the evolutionary relationship of gut predominant bacteria of silkworm, the 16S rRNA gene sequences of predominant genera for CK-144-F, CK-144-M, CPV-144-F and CPV-
144-M were selected and performed Blast. Most similar 16S rRNA gene sequences of predominant genera were used to construct the phylogenetic trees. The abundance of a predominant genus was indicated in the phylogenetic tree. The topological structure of the tree for CK-144-F (Fig 5A) was similar to that for CK-144-M (Fig 5B), but the abundance of predominant genera in CK-144-F and CK-144-M was noticeably different. The topological structure and components of the tree for CPV-144-F (Fig 5C) were different compared to CPV-144-M (Fig 5D). The phylogenetic trees of the predominant genera in the control were different compared to the infected silkworms, indicating the composition and abundance of predominant genera were changed following infection with BmCPV. Differences in the change patterns for females and males were also observed (Fig 5).

Relevance analysis of abundance of the predominant genera
PCA analysis was used to investigate the relevance of abundance of the 15 predominant genera in the bacterial gut microbiota. These genera were distributed in three different quadrants (Fig 6). There was a positive correlation between the abundance of *Staphylococcus* (6), *Pseudomonas* (9), *Methylobacterium* (11) and *Acinetobacter* (13); and the abundance of other predominant bacteria genera (1, 3–5, 7, 8, 10, 12, 14 and 15) were showed positive correlation with each other. The abundance of *Enterococcus* (2) was correlated negatively with the abundance of the most predominant genera.
Discussion

Complex intestinal microbial communities were believed to provide some benefits to their host [20]. Human health can be influenced by intestinal microbes [21] and the composition, diversity and functions of intestinal bacteria received a great deal of attention. Insects are a very diverse group and it has been reported that the microbial community can contribute to insect adaptation [22, 23], heat tolerance [24], protection against pathogens or natural enemies [25, 26, 27], reproduction [28] and vector competence [29].

In previous study, the culture-dependent, PCR and 16S rDNA- RFLP (restriction fragment length polymorphism) methods were adopted to investigate the silkworm gut microbiota. Only bacteria of $10^{-16}$ genera could be isolated from the gut contents by the culture-dependent method [1], 14 genotypes bacteria were detected by 16S rDNA- RFLP method [30] and 14 genera were found by PCR method [5]. However, compared with the traditional methods,
Pyrosequencing was applied and 199 genera in the intestinal contents of healthy fifth instar silkworm larvae were indentified, which provided adequate detailed information about silkworm gut microbiota. Rarefaction analysis showed that the sequencing approach was not carried out sufficiently to reach a plateau in this study, indicating the true bacterial diversity in the silkworm gut was underestimated.

Till date, various types of bacteria have been identified in the intestinal contents of insects. The gut bacterial community of the oriental armyworm (Mythimna separata) has been investigated; bacteria belongs to Cyanobacteria, Firmicutes, Actinobacteria, Gracilicutes and Proteobacteria genera were ubiquitous in the gut content [31]. Wild populations of Aedes albopictus and Aedes aegypti have been shown to harbor principally Proteobacteria and Firmicutes, including the Acinetobacter, Asaia, Delftia, Pseudomonas, Wolbachia and Bacillus genera, as well as members of the family Enterobacteriaceae [32]. Bacteria of 16 phyla including Proteobacteria and Firmicutes were found in the gut contents of the domesticated silkworm in the present study. Members of the Proteobacteria and Firmicutes phyla are present in armyworms, mosquitoes and silkworms, suggesting the intestinal bacterial microbiota of insects share similar characteristics; however, there are differences in the composition and diversity of bacterial microbiota in different insects, stated that the diversity of gut bacteria in insects can be affected by environment, habitat, diet and developmental stage [33]. Mutualisms between microbes and insects are ubiquitous [34]. The symbiotic relationship between termites and spirochetes was established more than 2000 million years ago [35], the fitness of the bean bug Riptortus clavatus can be increased by the symbiotic Protobacteria Burkholderia spp., so we speculated the composition and abundance of the predominant gut bacteria in the silkworm is the result of co-adaptation and co-evolution between the silkworm host and intestinal bacteria.

It was reported that the predominant genera in the bacterial microbiota of different silkworm strains were Brevundimonas, Stenotrophomonas, Enterobacter and Staphylococcus in strain Dongting × Bibo [30]. Enterococcus and Thermus were the predominant genera in the C108 and SCN2 strains; however, Enterobacter was not found [28]. In the present study, the
predominant genera in strain Daizo were *Enterococcus*, *Delftia*, *Ralstonia*, *Pelomonas*, *Tepidimonas*, *Aurantimonas*, *Pseudomonas*, *Aspromonas* and *Staphylococcus*, which showed there was an obvious difference in composition of the intestinal bacteria between different silkworm strains. Previous studies revealed the diversity of locust gut bacteria protects against pathogen invasion [36] and Chromobacterium Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities [37], so we conjecturing the composition of the intestinal bacterial community might be an important factor resulting in differences of resistance to pathogens between silkworm strains. The pH of the digestive juice, which can be impacted by intestinal bacteria, was involved in the resistance of silkworms to pathogens. *Enterococcus* was a predominant genus in the silkworm gut bacteria. Some species of *Enterococcus* produce acetate and its accumulation might reduce the pH of digestive juice in the grasshopper [38]. *Enterococcus faecalis*, a predominant species of intestinal bacteria in silkworm was commonly found at alkaline pH (8–9) and acidifies its environment through its metabolism [39]. Reducing the pH of the digestive juice can protect an insect from attack by a poisonous parasporal crystal of *Bacillus thuringiensis* or infection with pathogens. The silkworm can be infected only after the virion embedded in the polyhedral bodies of *B. mori* nucleopolyhedrovirus (BmNPV) or cypovirus are released at higher pH values. Germination of the microsporum *N. bombycis* [40] and activation of *Bacillus thuringiensis* δ-endotoxin required alkaline conditions [41], suggesting the abundance of *Enterococcus* spp. in the bacterial microbiota was involved in the resistance of silkworm to BmNPV, BmCPV and *N. bombycis*. Nevertheless, whether metabolic products of the intestinal bacteria can also directly inhibit infection of the silkworm by pathogens deserves further exploration.

Insect intestinal bacteria were involved in digestion and nutrient uptake [42], the composition and diversity of the silkworm intestinal microbiota were showed impact by forage [5]. In the present study, we found the composition and diversity of intestinal microbiota were noticeably reduced at the later period of the fifth instar (144 h of the fifth instar). Food consumption was reduced at the later period of the fifth instar, and the silkworm eventually stopped eating and empties the intestinal content before cocooning, so the decrease of composition and diversity of intestinal microbiota at 144 h of the fifth instar was related to empty the intestinal content for metamorphosis of the silkworm.

Sex differences in the gut microbiome found in the mouse drives hormone-dependent regulation of autoimmunity [43], and sex differences in the immunocompetence and susceptibility to pathogens have been also observed in different insect groups [44, 45]. Both males and females were able to enhance survival in the adult stage as a result of being injected bacteria at the larval stage; it was due to differential gender immune response [46]. According to Ryan evidence, gut symbionts influenced diet selection of male and female *Gryllus pennsylvanicus* differently, and also recommended that sex-specific dietary selection may be because of the fact that male and female crickets have different nutritional requirements [47]. Usually, the resistance to pathogens and feed utilization efficiency for the male silkworms were higher than female silkworm. In our investigation, we found the composition and diversity of gut bacterial microbiota in the silkworm was different between females and males. We speculated the difference of resistance and feed utilization efficiency between genders could be a result of differences in the gut microbiome.

The resistance of insects to pathogens is influenced by intestinal bacteria; conversely, the composition and diversity of intestinal bacteria are influenced by infection with pathogen. Silkworm cytoplasmic polyhedrosis is usually associated with bacterial gut disease. In the current study, we found bacterial diversity in the gut content was decreased after infection with BmCPV and the abundance of bacteria changed noticeably with the course of BmCPV infection. The abundance of *Enterococcus* spp. at 144 h post infection increased to 88.75%, which
was 1.28-fold more than control and the abundance of *Pseudomonas* spp. decreased to 0.02%, which was 27-fold lower compared to untreated batch. Our finding suggested that homeostasis of the intestinal microbiota might be broken by infection with BmCPV, which initiated generation of bacterial gut disease of the silkworm. Some *Enterococcus* spp. were opportunistic pathogens of silkworm [48,49], the number of bacteria of the genus *Enterococcus* was increased in silkworms infected with *N. bombycis*[6], a similar result was observed in this study, which suggested that the increase of *Enterococcus* spp related to the immune response in the silkworm.

A recent investigation revealed an antibacterial peptide cecropins gene expression level in silkworm was upregulated after infection with BmCPV [50]. Gram-negative bacteria were generally more sensitive to cecropins than Gram-positive organism [51]. In the present study, abundances of the predominant genera *Enterococcus* and *Staphylococcus* belonging to Gram-positive bacteria were roughly increased and copiousness of the predominant genera belonging to Gram-negative bacteria was decreased after infection with BmCPV infection. Therefore, it is suggested that observed changes in relative abundance was related to the upregulation of cecropins after infection with BmCPV.

Co-existence and competition relationship between gut bacteria were found in the silkworm. The abundance of *Staphylococcus, Pseudomonas, Methylobacterium* and *Acinetobacter* were in positive correlation with each other, while the abundance of *Enterococcus* spp. was in negative correlation with the most predominant genera, implying that resistance of the silkworm to infection with pathogens can be increased by the use of probiotics and optimization of the gut bacterial microbiota.

Some species of *Enterococcus* were probiotic and beneficial to healthy of host. *Enterococcus faecalis* CECT7121 is a probiotic strain that has been demonstrated to implant itself, persist and induce protective immune responses in several biological models [52,53,54]. Use the liquid probiotic form *Enterococcus faecalis* L3 in infants had a positive impact on overall health and can increase resistance to acute respiratory infections [55]. In insect, previous studies indicated that intestinal bacteria inhibit the growth of *Bacillus thuringiensis* in the larvae of the oriental tea tortrix, *Homona magnanima* [56] and the substance secreted by *Enterococcus* inhibit the germination of *N. bombycis* spores [57], and furthermore, genus *Enterococcus* is the most dominant bacteria in the silkworm intestinal microflora, therefore, we speculated that *Enterococcus* can be used as probiotics to defense against pathogen invasion.

**Supporting Information**

**S1 Table.** Proportion of genera in the intestinal bacterial community at different time points in the fifth instar of healthy and BmCPV-infected silkworms. CPV were genera detected after infection with BmCPV. 24,72 and 144 represent the gut contents were respectively collected at 24,72 and 144 h in the fifth instar. The original data of pyrosequencing related to this article can be found in GenBank.

(DOCX)

**S2 Table.** Accession numbers of original data. CK (CPV)-24(72,144)-F (M) are samples mentioned in Table 1.

(DOCX)

**Author Contributions**

Conceived and designed the experiments: CLG RYX GLC XLH ZLS. Performed the experiments: ZLS YHL HZ DK. Analyzed the data: CLG ZLS YHL HZ DK BL YCG MZ ZL FC SLK
LYZ. Contributed reagents/materials/analysis tools: CLG ZLS RYX GLC XLH YHL. Wrote the paper: CLG ZLS.

References
1. Yuan ZH, Lan XQ.-q., Yang T, Xiao J, Zhou ZY. Investigation and analysis of the bacteria community in silkworm intestine. Acta Microbiologica Sinica, 2006; 46(2): 285–29 PMID: 16736593
2. Sun XQ, Huang XY, Dong CJ, Liu ZH, Zheng CW. Silkworm intestinal part aerobic and facultative anaerobic microorganisms and the study of silkworm with probiotics. Sichuan silk, 1996; 24 (1): 13–15
3. Lu XM, Jin W, Qian YH, Gong CL. Distribution of the Enterococci Isolated from the intestine of the Silkworm, Bombyx mori. Science of Sericulture. 1999; 29(3): 158–162
4. Schmidt TM, Relman DA Phylogenetic identification of uncultured pathogens using ribosomal RNA sequences. Methods Enzymol. 1994; 235: 205–222 PMID: 7520119
5. Xiang YQ, Wang XQ, Feng W, et al. Comparative analysis of the composition of dominant intestinal microflora in silkworm reared with different forages. Acta Ecologica Sinica. 2010; 30(14): 3875–3882
6. Lu XM, Huang SK, Wang FW. Distribution of the Enterococci isolated from intestine of the pebrine infected silkworm. Science of Sericulture. 2003; 29(2):151–156
7. Dennis PG, Guo K, Imelfort M, Jensen P, Tyson GW, Rabaey K. Spatial uniformity of microbial diversity in a continuous bioelectrochemical system. Bioresearch Technol, 2013; 129, 599–605. doi: 10.1016/j.biortech.2012.11.098 PMID: 23313735
8. Li S, Chou HH. Lucy2: an interactive DNA sequence quality trimming and vector removal tool. Bioinformatics, 2004; 20(16): 2865–2866 PMID: 15130926
9. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 2009, 37,D141–145 doi: 10.1093/nar/gkn879 PMID: 19004872
10. Schloss P D. Evaluating different approaches that test whether microbial communities have the same structure. ISME J, 2008; 2(3), 265–275. doi: 10.1038/ismej.2008.5 PMID: 18239608
11. Motegi C, Nagata T, Miki T, Weinbauer MG, Legendre L, Rassoulzadegan F, et al. Interactive Effects of Viral and Bacterial Production on Marine Bacterial Diversity. PLoS One, 2013; 8(11), e76800. doi: 10.1371/journal.pone.0076800 PMID: 24244268
12. Shannon CE. A mathematical theory of communication. ACM SIGMOBILE Mobile Computing and Communications Review.2001; 5(1): 3–55.
13. ter Braak C.T.F., and Smilauer P. CANOCO Reference Manualand User’s Guide to Canoco for Windows. Software for Canonical Community Ordination(Version4). Wageningen: Centre of Biometry. 1998.
14. wei Xue. Statistical analysis methods and applications of Statistical Product and Service Solutions. Publishing House Of Electronics Industry, Beijing 2009; 34–35
15. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QiIME allows analysis of high-throughput community sequencing data. Nat Methods, 2010; 7(5): 335–336. doi: 10.1038/nmeth.f.303 PMID: 2083131
16. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4: 406–425 PMID: 3447015
17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729 doi: 10.1093/molbev/ms31017 PMID: 24132122
18. Martin AP. Phylogenetic Approaches for Describing and Comparing the Diversity of Microbial Communities. Appl Environ Microbiol, 2002; 68(8), 3673–3682. PMID: 12147459
19. Cole JR, Chai B, Farris RJ, Wang Q, Kulaq LM, McGarrell DM, et al. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput tRNA analysis. Nucleic Acids Research, 2005; 33, 294–296.
20. Flint HJ, Bayer E A, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol, 2008; 6(2):121–131. doi: 10.1038/ nmicro1817 PMID: 18180751
21. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Wang J. (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature, 464(7285), 59–65. doi: 10.1038/ nature08821 PMID: 20203603
22. Moy A, Pereto J, Gil R, & Lalrome A. Learning how to live together: genomic insights into prokaryote-animal symbioses. Nat Rev Genet, 2008; 9(3), 219–229. doi: 10.1038/nrg2319 PMID: 18268909
23. Oliver KM, Degnan PH, Burke GR, Moran NA. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annual Review of Entomology, 2010; 55, 247–266 doi: 10.1146/annurev-ento-112408-085305 PMID: 19728837

24. Montllor CB, Maxmen A., Purcell AH. Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. Ecological Entomology, 2002; 27(6), 189.

25. Hedges LM, Brownlie JC, O’Neill SL, Johnson KN. Wolbachia and virus protection in insects. Science, 2008; 322(5902):702. doi: 10.1126/science.1162418 PMID: 1897434

26. Oliver KM, Degnan PH, Hunter MS, Moran NA. Bacteriophages encode factors required for protection in a symbiotic mutualism. Science, 2009; 325(5943):992–4. doi: 10.1126/science.1174463 PMID: 19969350

27. Scarborough CL, Ferrari J., Godfray HC. Aphid protected from pathogen by endosymbiont. Science, 2005; 310(5755):1781. PMID:16357252

28. Xiang H,Li MW,Zhao Y, Zhao LP, Zhang YH, Huang YP. Bacterial community in midguts of the silk-worm larvae estimated by PCR/DGGE and 16S rDNA gene library analysis. Acta Entomologica Sinica. 2007; 50(3):222–233

29. Gottlieb Y, Zchori-Fein E, Mozes-Daube N, Kontsedalov S, Skaljac M, Brumin M, et al. The transmission efficiency of tomato yellow leaf curl virus by the whitefly Bemisia tabaci is correlated with the presence of a specific symbiotic bacterium species. J Virol, 2010; 84(18):9310–9317. doi: 10.1128/JVI.00423-10 PMID: 20631135

30. Tian ZH, Hui FL, Ke T, Kan YC, Wen ZZ. Molecular Analysis of the Bacteria Community Composition in Silkworm Midgut. Sericultural science magazine, 2007; 33(4):592–595

31. He C, Nan X, Zhang Z, and Li M. Composition and diversity analysis of the gut bacterial community of the Oriental armyworm, Mythimna separata, determined by culture-independent and culture-dependent techniques. J Insect Sci, 2013; 13, 165. doi: 10.1673/031.013.16501 PMID: 24773514

32. Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LH, Ravelonandro P, et al. Bacterial diversity of field-caught mosquitoes, Aedes albopictus and Aedes aegypti, from different geographic regions of Madagascar. FEMS Microbiol Ecol, 2011; 75(3), 377–389. doi: 10.1111/j.1574-6941.2010.01012.x PMID: 21175696

33. Yun JH, Roh SW, Whon TW, Jung MJ, Kim MS, Park DS, et al. Insect Gut Bacterial Diversity Determined by Environmental Habitat, Diet, Developmental Stage, and Phylogeny of Host Appl Environ Microbiol, 2014; 80(17):5254–5264

34. Sabree ZL, Moran NA. Host-specific assemblages typify gut microbial communities of related insect species, SpringerPlus, 2014; 3:138 http://www.springerplus.com/content/3/1/138 PMID: 24741474

35. Wier A1, Dolan M, Grimaldi D, Guerrero R, Wagensberg J, Margulis L. (2002) Spirochete and protist symbionts of a termite (Mastotermes electrodominicus) in Miocene amber[J]. Proc Natl Acad Sci USA, 2002; 99(3):1410–1413 PMID: 11818534

36. Dillon RJ, Vennard CT, Buckling A, Chamley AK. Diversity of locust gut bacteria protects against pathogen invasion. Ecol Lett, 2005; 8(12):1291–1298

37. Ramirez JL, Short SM, Bahia AC, Saraiva RG, Dong Y, Kang S et al. Chromobacterium Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities. PLoS Pathog, 2014; 10(10)

38. Mead LJ, Khachatourians GG, Jones CA. Microbial Ecology of the Gut in Laboratory Stocks of the Migratory Grasshopper, Melanoplus sanguinipes (Fab.) (Orthoptera: Acrididae). Appl Environ Microbiol, 1988; 54(5):1174–1181 PMID: 16347630

39. Manero A, Blanch AR. Identification of Enterococcus spp. with a biochemical key. Appl. Environ. Micobiol, 1999; 65(10):4425–4430

40. Lu XM,Wang FW. Inhibition of Cultured Supernatant of Enterococci Strains on Germination of Nosema bombycis Spores in vitro. Science of Sericulture. 2002; 28(2):126–128

41. Wilson GR, and Benoit TG. Alkaline pH activated Bacillus thuringiensis spores. 1993; 62, 87–89.

42. Brummel T, Ching A, Seroude L, Simon AF, Benzer S, Drosophila lifespan enhancement by exogenous bacteria. Proc Natl Acad Sci USA, 2004; 101(35):12974–12979 PMID: 15322271

43. Markle JG, Frank DN, Mortin-Tooth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity[J]. Science, 2013; 339(6123):1084–1088 doi: 10.1126/science.1233521 PMID: 23328393

44. Adamo SA, Jensen M, Younger M. Changes in lifetime immunocompetence in male and female Gryllus texensis (formerlyG-integer): trade-offs between immunity and reproduction. Anim Behav. 2001; 62:417–425
45. Rantala MJ, Roff DA. Analysis of the importance of genotypic variation, metabolic rate, morphology, sex and development time on immune function in the cricket, Gryllus firmus. J Evol Biol. 2006; 19:834–843. PMID: 16674580

46. Moreno-García M, Vargas V, Ramírez-Bello I, Hernández-Martínez G, Lanz-Mendoza H. Bacterial Exposure at the Larval Stage Induced Sexual Immune Dimorphism and Priming in Adult Aedes aegypti Mosquitoes. PloS one, 2015; 10(7):e0133240 doi: 10.1371/journal.pone.0133240 PMID: 26181517

47. Schmid RB, Lehman RM, Lundgren JG. Sex-Specific Interactions of Microbial Symbioses on Cricket Dietary Selection. Environmental Entomology. 2014; 43(4): 896–902 doi: 10.1603/EN13311 PMID: 24914929

48. LYSENKO O. ‘Streptococcus bombycis’, its Taxonomy and Pathogenicity for Silkworm Caterpillars. J. Gen. Microbiology, 1958; 18, 7741–7781.

49. Kodama R, N. Y. B. e. i. f. s. l. I. The pathogenic effects of two isolates on aseptically reared silkworm larvae. J of Seri cultural Science of Japan, 1968; 37, 477–482.

50. Kolliopoulou A, Van Nieuwerburgh F, Stravopodis DJ, Deforce D, Swevers L, Smagghe G, et al. Transcriptome Analysis of Bombyx mori Larval Midgut during Persistent and Pathogenic Cytoplasmic Polyhedrosis Virus Infection. PLoS ONE, 2015; 10(3): e0121447. doi: 10.1371/journal.pone.0121447 PMID: 25816294

51. Moore AJ, Beazley WD, Bibby MC, Devine DA. Antimicrobial activity of cecropins. J Antimicrob Chemother. Jun, 1996; 37(6):1077–89. PMID: 8836811

52. Castro MS, Molina MA, Sparo MD, Manghi MA. Effects of Enterococcus faecalis CECT7121 on the specific immune response after DTPw vaccinatation. Int J Probiotics Prebiotics, 2008; 3(1):25–30.

53. Castro MS, Molina MA, Di Sciullo P, Azpiroz MB, Leocata Nieto F, Sterín-Speziale NB, et al. Beneficial activity of Enterococcus faecalis CECT7121 in the anti-lymphoma protective response. J Appl Microbiol, 109(4):1234–1243. doi: 10.1111/j.1365-2672.2010.04747.x PMID: 20477887

54. Castro MS, Azpiroz MB, Molina MA, Moutere AC, Alaniz FS, Maldonado AM, et al. Preliminary studies on the prevention of the ovalbumin-induced allergic response by Enterococcus faecalis CECT7121 in mice. Int Arch Allergy Immunol, 2012; 157(1):11–20. doi: 10.1159/000324673 PMID: 21894024

55. Gonchar NV, Suvorov AN, Maryshev VP, Sorokina TM, Churkova TV, Kharit SM. [PROBIOTICS, NUTRITIONAL STATUS AND RESISTANCE TO RESPIRATORY INFECTIONS IN INFANTS], Eksp Klin Gastroenterol. 2015;(1):48–54. PMID: 26281160

56. Jun Takatsuka J, Kunimi Y. Intestinal Bacteria Affect Growth of Bacillus thuringiensis in Larvae of the Oriental Tea Tortrix, Homona magnanima Diakonoff (Lepidoptera: Tortricidae). J Invertebrpathol. 2000; 76(3): 222–226

57. Wang FW, Lu XM, Huang SK. Kinetic studies of Enterococci inhibition on the germination of Nosema bombycis spores, Science of Sericulture. 2003; 29(2):157–161