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Isolation and characterization of enzyme-producing bacteria of the silkworm larval gut in bioregenerative life support system

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ABSTRACT

Silkworm (*Bombyx mori* L.) larvae were used as an ideal animal protein source for astronauts in bioregenerative life support system (BLSS). Here, we compared the differences in bacterial communities of the silkworm larval gut and cellulase-producing and amylase-producing bacteria between the BLSS rearing way (BRW) and the traditional rearing way (TRW) through Illumina Miseq sequencing, culture-dependent approach, 16S rDNA and ITS sequencing, phylogenetic analysis to find the role of gut bacteria in food digestion. The analysis of Miseq showed that the gut microbiota in the BRW was significantly changed than that in the TRW. Results revealed that the isolates can produce cellulose-degrading and starch-degrading enzymes of gut bacteria of silkworm in the BRW which decreased compared with that of the TRW, but the number of isolates both secrete cellulase and amylase are equal. The isolates that can produce both enzymes in the TRW were *Alternaria sp. Preussia sp* and *Coprinellus radians.* Meanwhile, in the BRW we found *Enterococcus*, *Erwinia* and *Pantoea* can produce cellulase and amylase. We could use the dominant populations to make probiotic products for nutrient absorption and disease prevention in BLSS to improve gut microecology, as well as the yield and quality of animal protein.

1. Introduction

The insect gut is inhabited by a wide diversity of microorganisms as a result of its constituting intestinal microbial ecosystem. The gut microbiota is involved in the host's digestion, nutrition, development, resistance to pathogens invasion. The composition and structure of microbial are dynamic, which can be varied with changing nutrient availability, physiological environments, and the proximity to other organisms [1,2]. Loss of microorganisms often results in abnormal development and reduces survival of the insect host [3,4]. Based on the theory of microecology, insect relying on gut microbes provides a variety of digestive enzymes, to complete its food digestion, nutrient absorption and metabolism [5]. However, few have discussed the possibility that microorganisms may produce some of the digestive enzymes to provide essential nutrients or assist in important metabolism function related to host food ingestion [1].

Mulberry silkworm (*Bombyx mori L.*) is an important economical insect whose importance is reflected not only by its silk production but also by its valuable nutritional

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composition. The idea of rearing mulberry silkworm larvae to provide animal protein for crew in bioregenerative life support system (BLSS) required by long-term missions to the moon and Mars is widely accepted. This is because silkworm has many positive merits such as high protein content, reasonable nutrient compositions and ample contents, a short lifespan, easy breeding method, small growth room, and little odor and wastewater produced [6]. Due to limitation of space and resource, mulberry silkworm rearing method in BLSS was different from traditional rearing method which only uses mulberry leaves. In BLSS, mulberry silkworms of the first three instars (from the 1st day to 16th day) were fed with mulberry leaves and for those of the last two instars (from the 17th day to 25th day) were fed with lettuce leaves [7]. In this rearing way, the yield and the growth rate of silkworm larvae reared using this method were a little lower than those feeding on mulberry leaves purely. Prior study has provided evidence that gut microbiota of silkworms fed on lettuce leaves was relatively simple. The appearance of profitless bacteria in the gut of silkworm under the BLSS rearing way might break down the balance structure of healthy gut microbial community, resulting in reduced digestive enzyme activity [8]. It has been reported that many silkworm intestinal bacteria produce digestive enzymes like amylase and cellulose [9]. Thus, it is potentially possible that the change of gut flora due to lettuce leaf feeding may contribute to the decrease of physiological activity and cause death of the silkworm. However, how diet compositions in BLSS shape gut enzyme-producing bacteria is still unknown.

In this study, the gut bacterial diversity and enzyme-producing bacteria of silkworm larvae reared with the traditional rearing way (TRW) and BLSS rearing way (BRW) were investigated using culture dependent, culture-independent and Illumina Miseq approaches. The changes of cellulase-producing bacteria, amylase-producing bacteria in silkworm larvae in response to lettuce leaf feeding were revealed. This study may promote the development of probiotic products of animal protein under BLSS.

2. Materials and methods

2.1. Silkworm strains and rearing methods

The silkworm eggs *B. mori* L. 872 × 871 were bought from Guangtong Silkworm seed Co. Ltd. (Shandong Province, China). The silkworm eggs were incubated under a 12-h light/12-h dark cycle in an artificial cultivation box at 25 °C and 80% of relative humidity. When 20% of eggs had little black dots on the surface, they were shaded with black gobo for about 48 h to ensure the larvae hatching out at one time. The silkworm larvae were reared with mulberry leaves from the first to third instar and then divided into two groups: the BLSS breeding group reared with stem lettuce leaves and the conventional breeding group still reared with mulberry leaves at the beginning of the fourth instar [8].

2.2. Isolation of gut bacteria and DNA extraction

When the two groups of silkworms grew to the third day of the fifth instar, 10 individuals of each group were selected and subjected to starvation overnight. Those silkworms were surface decontaminated by wiping with 70% ethanol solution and scorched gently in a flame. The content of gut was taken and placed into sterile microcentrifuge tubes on ice under aseptic condition. According to the screening standard of bacteria, tenfold serial dilution was spread and incubated for inoculation. On each nutrient agar plate, 0.1 mL of intestinal content of two groups was spread and incubated at 37 °C for 2 days. All samples were repeated three times. The media used for the isolation of bacteria included nutrient agar, potato dextrose agar, Gause's No. 1 agar medium, which were autoclaved at 121 °C for 15 min, and pH value was adjusted to 9.2–9.8 [10]. Colonies of each group were picked out, purified three times by inoculating on the corresponding agar plates, and further transferred to agar slants.

Pooled DNA samples of the fifth instar in the BLSS and the conventional breeding groups were composed of DNA extracted from the selected 10 individuals. DNA was extracted with a Promega DNA Kit (Promega, USA), quantified with a BioPhotometer (Eppendorf), and stored at −20 °C until used.

2.3. Illumina Miseq sequencing and data analysis

High-throughput sequencing was conducted at Majorbio Co., Ltd. (Shanghai, China). The bacterial 16S rRNA gene was amplified with primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) targeting the V3–V4 region (about 470 bp). The fungal 18S rRNA gene was amplified with primers ITS1-1737F (GGA AGT AAA AGT CGT AAC AAGG) and ITS2-2043R (GCT GCG TTC TTC ATC GAT GC) targeting the ITS1–ITS2 region (about 246 bp). The PCR amplification was conducted using specific primers with barcode and high fidelity TrashStart Fastpfu DNA Polymerase (TransGen Biotech, China). PCR amplification was performed in a total volume of 20 μL containing 4 μL 5 × FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL 5 μM primers, 0.4 μL FastPfu Polymerase and 10 ng DNA template. The bacterial 16S rRNA gene PCR thermal cycle profile was as follows: 2 min at 95 °C; 28 cycles of 30 s at 95 °C, 30 s at 61 °C, 45 s at 72 °C; final 10 min at 72 °C, and cooling at 10 °C. The fungal 18S rRNA gene PCR profile was similar to the bacterial profile except that it had five more cycles.

The Miseq sequencing was collecting the fluorescence signal to read the sequence of DNA fragment. All sequences were divided depending on the similarity level and statistical analysis of biological information under 97% similar level of OTU. The community structure was analyzed statistically at different classification levels and visual analysis of community structure and phylogeny finally.

2.4. Screening of cellulase-producing bacteria, amylase-producing bacteria

Each isolate was inoculated on carboxy methyl cellulose (CMC) agar plate medium (0.1%CMC–nutrient agar,