Establishment of a gnotobiotic silkworm model

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Summary Gnotobiotic animals are useful for investigation of the effects of specific lactic acid bacteria on individual animals. Here we report that lactic acid bacteria colonize and proliferate in the intestinal tract of germ-free silkworms. When silkworms hatching from formalin-treated eggs were reared to fifth-instar larvae with an artificial diet containing antibiotics, bacteria and fungi were not observed in their intestines. An antibiotic-free diet supplemented with viable lactic acid bacteria, such as Enterococcus faecalis 0831-07, Lactococcus lactis 11/19-B1, or Leuconostoc carnosum #7-2, was fed to the germ-free silkworms for 1 day. After feeding the larvae on a diet without lactic acid bacteria for 5 days, each type of lactic acid bacterium was found in the intestine. Moreover, an increase in the number of Enterococcus faecalis 0831-07 was observed in the intestine 2-5 days after ingestion. These findings suggest that we successfully established a method to construct a gnotobiotic silkworm model.

Keywords: Gnotobiotic animal, lactic acid bacteria, silkworm

1. Introduction

Lactic acid bacteria used for various fermented foods, such as yogurt and pickles, are considered to contribute to human health (1-3). Some lactic acid bacteria are reported to colonize in the mammalian intestinal tract to form intestinal microbiota (4,5).

In general, mammals are used to evaluate the effects of lactic acid bacteria for promoting health and preventing disease (4,5). The influence of lactic acid bacteria and intestinal bacteria on the host is often studied using gnotobiotic animals in which specific bacterial species are maintained as viable bacteria in the intestinal tract (6-11). The use of gnotobiotic animals allows for investigation of the effects of specific bifidobacterium and lactic acid bacteria on individual animals without confounding by other intestinal microbiota (12). Gnotobiotic mammalian animals are expensive, however, and special equipment and facilities are required to rear them. Furthermore, utilizing a large number of mammals raises ethical problems from the viewpoint of animal welfare. To overcome the problems associated with the use of mammals, insects such as fruit flies, honey bees, and cockroaches have been proposed as gnotobiotic animals (8,10,11). These animals, however, are too small for injecting sample solutions with syringes and collecting their blood for biochemical analysis. We established silkworm infection models and diabetes models for exploring candidate functional foods and pharmaceuticals (13-17). Silkworms are associated with lower costs and fewer ethical problems compared with mice, yet their size is sufficiently large for injection experiments (13,14,16-21). We successfully obtained useful lactic acid bacteria using the silkworm infection and diabetes models (22-25), although a method for constructing gnotobiotic silkworms has not been established.

In this study, we report that lactic acid bacteria colonize and proliferate in the silkworm intestinal tract. The gnotobiotic silkworms we developed are expected to be useful for evaluating the effects of lactic acid bacteria.

2. Materials and Methods

2.1. Silkworm rearing conditions

Silkworms were reared according to the previously reported method (24). Fertilized silkworm eggs (Bombyx mori, Hu Yo x Tukuba Ne) were purchased from
We then examined whether orally administered lactic acid bacteria can survive in the silkworm intestinal tract for at least 5 days. Enterococcus faecalis, Lactococcus lactis, and Leuconostoc carnosum were administered orally to silkworms on the first day of the fifth-instar stage. The silkworms were then fed a diet without lactic acid bacteria for 5 days, and extracts of the intestinal tracts were prepared to measure the number of viable cells. Bacterial species were determined by sequencing analysis of the amplified DNA encoding 16S rRNA.

### 3. Results and Discussion

#### 3.1. Viability of orally administered lactic acid bacteria in the silkworm intestinal tract

In our laboratory, silkworm eggs are treated with formalin and the larvae are fed an artificial diet containing antibiotics. Under such rearing conditions, viable bacteria and fungi are not detected on agar plates containing antibiotics. Under such rearing conditions, viable bacteria and fungi are not detected on agar plates containing antibiotics. In the present study, we examined whether lactic acid bacteria can be recovered from the intestinal tract when silkworms are fed a diet supplemented with lactic acid bacteria (Table 1). Fifth-instar silkworms were fed with Enterococcus faecalis 0831-07, Lactococcus lactis 11/19-B1, or Leuconostoc carnosum #7-2 for 1 day. The silkworms were then fed a diet without antibiotics for 5 days, and the materials in the intestinal tracts were spread on BHI agar plates and incubated. A number of colonies formed on the agar plate. In the control experiments, silkworms were fed a diet supplemented with physiologic saline, and no colonies formed. Two colonies that formed on the plate were picked up, and the DNA sequence encoding bacterial 16S rRNA was amplified by colony polymerase chain reaction using universal primers. Species of the bacteria were determined by sequencing analysis of the amplified DNA.

#### 3.2. Growth of lactic acid bacteria in the silkworm intestinal tract

In our laboratory, silkworm eggs are treated with formalin and the larvae are fed an artificial diet containing antibiotics. Under such rearing conditions, viable bacteria and fungi are not detected on agar plates containing antibiotics. In the present study, we examined whether lactic acid bacteria can be recovered from the intestinal tract when silkworms are fed a diet supplemented with lactic acid bacteria (Table 1). Fifth-instar silkworms were fed with Enterococcus faecalis 0831-07, Lactococcus lactis 11/19-B1, or Leuconostoc carnosum #7-2 for 1 day. The silkworms were then fed a diet without antibiotics for 5 days, and the materials in the intestinal tracts were spread on BHI agar plates and incubated. A number of colonies formed on the agar plate. In the control experiments, silkworms were fed a diet supplemented with physiologic saline, and no colonies formed. Two colonies that formed on the plate were picked up, and the DNA sequence encoding bacterial 16S rRNA was amplified by colony polymerase chain reaction using universal primers. Species of the bacteria were determined by sequencing analysis of the amplified DNA.

### Table 1. Isolation of lactic acid bacteria from silkworm intestine

<table>
<thead>
<tr>
<th>Ingested bacteria</th>
<th>Viable cell number (CFU / intestine of silkworm)</th>
<th>Recovered bacteria from silkworm intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterococcus faecalis 0831-07</strong></td>
<td>1.4-3.8 × 10^6</td>
<td><strong>Enterococcus faecalis</strong></td>
</tr>
<tr>
<td><strong>Lactococcus lactis 11/19-B1</strong></td>
<td>0.9-5.0 × 10^6</td>
<td><strong>Lactococcus lactis</strong></td>
</tr>
<tr>
<td><strong>Leuconostoc carnosum #7-2</strong></td>
<td>0.5-1.5 × 10^6</td>
<td><strong>Leuconostoc carnosum</strong></td>
</tr>
<tr>
<td><strong>Saline (control)</strong></td>
<td>&lt;100</td>
<td>None</td>
</tr>
</tbody>
</table>

Silkworm larvae on the first day of the fifth-instar stage were fed for 1 day with a diet with saline or one of the following lactic acid bacteria: Enterococcus faecalis 0831-07 (2.3 × 10^7 cfu/larva), Lactococcus lactis 11/19-B1 (2.6 × 10^7 cfu/larva), or Leuconostoc carnosum #7-2 (7.3 × 10^7 cfu/larva). The silkworms were then reared on a diet without lactic acid bacteria for 5 days, and extracts of the intestinal tracts were prepared to measure the number of viable cells (n = 2/group). Bacterial species of two independent colonies on each agar plate were determined by sequence analysis of the amplified DNA encoding 16S rRNA.

Ehime sericulture incorporated company (Ehime, Japan). The eggs were treated with 4% formalin. Larvae hatched from the eggs were fed an artificial diet containing antibiotics (Silkmate 2S, Nihon Nosan Corporation, Tokyo, Japan) up to the fifth-instar stage at 25°C. Square dishes (type 2, Eiken Instruments) were used as rearing cages for the first and second-instar larvae, and plastic food packs (192 × 120 × 46 mm, Chuo Kagaku, Saitama) were used for rearing the larvae in the later stages. Larvae on the first day of the fifth-instar stage that were fasted since the fourth molt were used for the experiments. A diet containing lactic acid bacteria was prepared by mixing 15 μL of lactic acid bacteria culture and 1 g of antibiotic-free artificial food, Silkmate 2S (Katakura Industries Co., Ltd., Tokyo).

### 2.2. Culture of lactic acid bacteria

Enterococcus faecalis 0831-07 (26), Lactococcus lactis 11/19-B1 (22), and Leuconostoc carnosum #7-2 (25) were streaked on deMan, Rogosa, and Sharpe (MRS) agar (Becton, Dickinson and Company, MD, USA) plates, and incubated at 30°C under anaerobic conditions. Bacterial colonies were isolated and further cultured to full growth in MRS broth (Becton, Dickinson and Company) at 30°C for 2-3 days under anaerobic conditions.

### 2.3. Measurement of viable cell number in the silkworm intestinal tract

The entire intestinal tract was aseptically isolated, chopped with scissors in 10 mL saline, homogenized, and the supernatant obtained. The supernatant was diluted in saline, and a 100-μL aliquot was spread on Brain Heart Infusion (BHI; Becton, Dickinson and Company, MD, USA) agar plates. After incubation at 30°C for 2 days, the number of colonies was counted and the number of viable cells in the sample was calculated.

### 2.4. Identification of bacteria

Bacterial species were identified by sequencing genes encoding 16S rRNA according to a previously reported method (24). Colonies that formed on BHI agar plates were picked up, and the DNA sequence encoding bacterial 16S rRNA was amplified by colony polymerase chain reaction using universal primers. Species of the bacteria were determined by sequencing analysis of the amplified DNA.
In this study, we produced a gnotobiotic animal using silkworms. Silkworms have several advantages as animal models, such as lower costs and fewer ethical problems as experimental animals. Silkworm infection and diabetes models are established, and the gnotobiotic silkworm model might be useful for screening for lactic acid bacteria with the potential to prevent or cure infectious diseases and diabetes.

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References


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