Brief Report

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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

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Table 1. Isolation of lactic acid bacteria from silkworm intestine

Ingested bacteria	Recovered bacteria from silkworm intestine	
Bacteria species	Viable cell number (CFU / intestine of silkworm)	Bacterial species
Enterococcus faecalis 0831-07	1.4-3.8 × 10^8	*Enterococcus faecalis*
Lactococcus lactis 11/19-B1	0.9-5.0 × 10^6	*Lactococcus lactis*
Leuconostoc carnosum #7-2	0.5-1.5 × 10^5	*Leuconostoc carnosum*
Saline (control)	< 100	None

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 × 10^6 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.

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 when the materials in the silkworm intestinal tract are spread and incubated (*27*). 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 plates were picked up, and the DNA region encoding 16S ribosomal RNA was sequenced. The results demonstrated that the two independent colonies were the lactic acid bacterial species added to the diet. The result suggests that lactic acid bacteria supplemented in the diet can survive in the silkworm intestinal tract for at least 5 days.

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

We then examined whether orally administered lactic

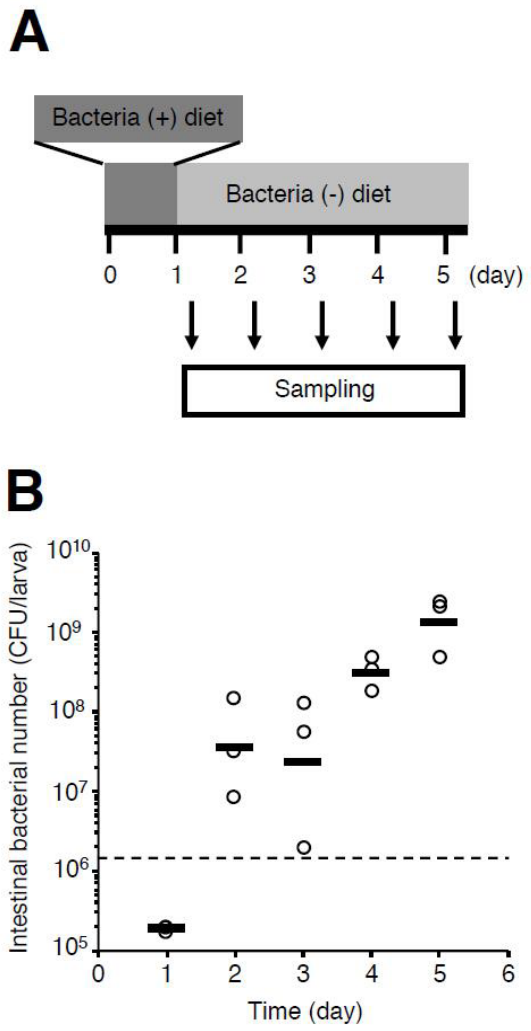

Figure 1. Growth of *Enterococcus faecalis* 0831-07 in intestinal tracts of silkworms. (A) Schematic of the experiment schedule. **(B)** Silkworm larvae on the first day of the fifth-instar stage were fed a diet with saline or *Enterococcus faecalis* 0831-07 (1.3×10^6 cfu/larva, shown by dashed line in the figure) for 1 day. Then, the silkworms were reared on a diet without lactic acid bacteria or antibiotics for 5 days, and the viable cell numbers in the intestinal tract were measured over time. Bars in the figure show the mean (n = 3/group).

acid bacteria grow in the silkworm intestinal tract. Silkworms were fed a diet with *Enterococcus faecalis* 0831-07 (1.3×10^6 cfu/larva) for 1 day, then further fed a diet without lactic acid bacteria, and the numbers of viable bacteria in the intestinal tract were monitored over 5 days (Figure 1). The bacterial number decreased from 1.3×10^6 cfu/larva on the day of ingestion to 1.7-2.0×10^5 cfu/larva on the subsequent day. Thereafter, the number of bacteria increased and reached 0.5-2.4 $\times 10^9$ cfu/intestine on day 5. This value was 100 times higher than that of the bacteria fed to the silkworms, indicating that *Enterococcus faecalis* 0831-07 proliferated in the silkworm intestinal tract. This finding confirms that our experimental system to establish a single bacterial species in the silkworm intestinal tract was successful, thereby providing a gnotobiotic silkworm model.

4. Conclusion

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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