Original Article

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Identification and methods for prevention of *Enterococcus mundtii* infection in silkworm larvae, *Bombyx mori*, reared on artificial diet

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Summary **Previously, it was reported that *Enterococcus mundtii* (*E. mundtii*) was associated with flacherie disease of silkworm larvae reared on artificial diet. In this study, we report that *E. mundtii* was isolated from diseased silkworm larvae, and validated as a pathogenic bacterium of the animal. When silkworm larva was infected with 1.04×10^6 colony-forming units of *E. mundtii* via oral administration of diet, half population died within six days, indicating that the bacterium is pathogenic to silkworm. Less severe infection was found to cause anorexia and hamper the development of larvae. This pathogen was found to proliferate in both time- and dose-dependent manner in the gastrointestinal tract of the animal. The bacterium was isolated from powder of artificial diet made from mulberry leaves, and from mulberry leaves growing at a field. Minimum inhibitory concentration determination revealed that this bacterium was susceptible to tested antibiotics. Vancomycin treatment of diet significantly decreased the number of *E. mundtii* in intestine of silkworm larvae infected with the bacteria, compared to control. Furthermore, autoclaving or gamma ray irradiation of diet was also effective for exclusion of *E. mundtii* from the diet without the loss of its nutrient capacities. These results suggest that mulberry leaves used in making artificial diet for silkworm larvae is one of the sources of *E. mundtii* infection; and that antibiotic treatment, autoclaving or gamma ray irradiation of artificial diet can exclude the bacteria.**

Keywords: Drug candidates, bacterial proliferation, gamma ray irradiation, pathogenic infection.

1. Introduction

Recent advances in pharmacy and medicine have brought to fore the need to develop disease models with invertebrate animals for evaluation of therapeutic behavior of drug candidates and novel pharmaceuticals. We are proposing use of silkworm as a model animal for this purpose. Apart from low cost of rearing, low possibility of bio-hazard problems, and easy injection with syringes during experimental researches; silkworm larvae as a non-mammalian animal also eliminate the ethical problems involved in studying mammals in preclinical tests for drug candidates (*1-4*). We recently discovered new antibiotic, lysocin E, from the cultured supernatant of *lysobacter sp*, a soil bacterium, by monitoring the therapeutic effects in silkworm model infected with *Staphylococcus aureus* (*5*).

Artificial diet-based mass production of silkworm larvae is a critical step for use of silkworm models for drug screening. However, infectious disease from pathogens is usually a major challenge sometimes leading to under-development, poor productivity and death of larvae. There have been previous reports of infection of silkworm larvae by various pathogens *vis-à-vis*: *Metarhizium anisopliae* (*6*), *Alphabaculovirus* (*7*), *parasitic Nosema species* (*8*), and *Bombyx* densovirus type 1 (*9*). Similarly, *Enterococcus mundtii* (*E. mundtii*) has been shown to be directly associated with flacherie disease of the silkworm larvae reared on artificial diet (*10*). In this present study, we explored the origin of observed pathogenic infection in industrial

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and transgenic strains of silkworm larvae, used as a non-mammalian animal model in drug discovery, and possibility of preventing such infections. We identified *E. mundtii* as the infectious pathogen and found that it came from artificial diets made from mulberry leaves. To confirm, we successfully isolated the bacteria from the leaves. Further results suggested that antibiotic treatment; autoclaving and gamma ray irradiation of artificial diet could handle the infection problems.

2. Materials and Methods

2.1. *Microorganisms and chemicals*

All used microorganisms were obtained from laboratory stock. Chloramphenicol and tetracycline were purchased from Nacalai Tesque Incorporation (Chuo-ku, Tokyo, Japan); while ampicillin, vancomycin, Sodium Hydroxide; and kanamycin were purchased from Wako Pure Chemical Industries (Mie-gun, Mie, Japan); and Tokyo Chemical Industries Ltd (Chuo-ku, Tokyo, Japan), respectively. Brain Heart Infusion (BHI) and Mueller Hinton Broths were purchased from Decton, Dickinson and Company (Franklin Lakes, New Jersey, USA).

2.2. *Determination of Minimum inhibitory concentrations of antibiotics*

For Minimum inhibitory concentrations (MICs) determination, bacteria were visually examined in two fold serial dilutions of tested samples in Mueller Hinton Broth medium according to National committee for clinical laboratory standards (*11*). Cultures were incubated at 37°C for 24 hours. MICs were determined using broth dilution method as described previously (*12*). The MIC was defined as the lowest concentration of the assayed antimicrobial agent (drug) that inhibited the visible growth of the *E. mundtii*, compared with that of the drug-free control, using the 96-well micro-titer plate format.

2.3. *Silkworm larvae, artificial diet, diet powder and mulberry leaves*

Fertilized eggs of silkworm, *Bombyx mori* (Hu.Yo x Tukuba.Ne) were obtained from Ehime Sanshu, Ehime, Japan. Artificial diet (Silkmate 2S) and diet powder (Silkmate 2M) purchased from Nosan Corporation (Yokohama, Kanagawa, Japan) were used for the experiment. Eggs were surface sterilized following the established procedure for germ-free rearing of larvae (*13,14*). Hatched larvae were raised by feeding on Silkmate 2S at room temperature till fifth instar before used for experiments. Larvae were sacrificed for gut analysis between 72 h and 120 h from the beginning of the 5th instar. Before sacrificing larvae, a disinfection treatment on the surface of larval body was carried out: larvae were dipped in 70% ethanol for 10 seconds. Mulberry leaves were harvested from the garden of the Graduate School of Agricultural and Life Sciences, The University of Tokyo (Bunkyo-ku, Tokyo, Japan).

2.4. *Identification of bacteria*

To identify all bacteria strains, single colonies were Gram stained (*15*). Bacterial species were determined by sequencing and analysis of 16S Ribosomal Ribonucleic Acid (16S rRNA) as previously described (*16*), with modification as follows: The genomic DNA was extracted from the 50 mL liquid Brain Heart Infusion (BHI) broth medium according to previous protocols (*17,18*). The 16S rRNA fragment was amplified using selected universal primers (both forward and reverse) in a GeneAmp Polymerase Chain Reaction (PCR) System 9700 thermal cycler with the following programs: 30 sec at 94°C, 30 sec at 55°C, and 1.5 min at 72°C for 35 cycles. The PCR reactions were terminated at 72°C for 7 min and thereafter, cooled at 4°C. To ascertain the specificity of the PCR amplification; negative control (PCR mix without DNA template) and positive control (chromosomal DNA of *E. mundtii*) were included. Amplification was confirmed by electrophoretic analysis of 5 µL PCR reaction mixtures on a 1% agarose gel, and was screened by ChampGel gel image disposal system. The PCR product was purified using a QIAamp Column DNA Gel Extraction Kit purchased from Qiagen (Chuo-ku, Tokyo, Japan) according to the manufacturer's instruction. The 16S rRNA was sequenced, and then analyzed with GenBank by Blast software.

2.5. *Examination of the effects of autoclaving, gamma ray irradiation or infection of diet on growth of silkworm larvae*

To understudy the effects of diet infection on growth, larvae were fed with either infected (2 g mixed with 10 µL of *E. mundtii* full growth culture) or non-infected artificial diet at 27°C for 24 h. The feeding with non-infected diet continued daily till the 6th day, weight of silkworm was measured and photo shots taken. To probe into the effects of severe heat treatment of diet powder and gamma ray irradiation on growth of larvae; thirty larvae were randomly divided into three groups: A, B and C (n = 10). Groups A and B, were fed with autoclaved (Silkmate 2M heat-treated to 121°C for 20 min using High Pressure-Steam Standard Autoclaving machine) and gamma ray irradiated diet (both freshly obtained from the manufacturer). While group C was fed with normal diet obtained from the laboratory stock (but also supplied by the same manufacturer). Feeding with same kind of diet in same proportion continued for 5 days. Total weights of larvae in different groups were measured daily.

2.6. *Determination of median lethal dose (LD_{50}) of E. mundtii in silkworm by oral administration*

LD_{50} of *E. mundtii* via oral administration was determined as follows: 50 µL of various dilutions of *E. mundtii* full growth culture were adsorbed into 5 g diets and fed to 5 silkworm larvae. The number of colony-forming units (CFUs) of *E. mundtii* administered per larva at which 50% of the tested population was dead after 6 days of administration was defined as LD_{50}.

2.7. *Isolation of E. mundtii from mulberry leaves*

Mulberry leaves were crushed with a sterilized teflon homogenizer in sterilized physiological saline. The aliquot was streaked on BHI agar plates, and incubated at 30°C for 24 h.

2.8. *Data and statistical analysis*

Data were analyzed using Graph-pad prism student *t*-test. Descriptive data were expressed as mean ± Standard error of mean (SEM). $p < 0.0001$ was considered statistically significant.

3. Results

3.1. *Identification of E. mundtii from feces of weakened silkworm reared on artificial diet*

We occasionally experienced disease problems in our non-mammalian animal model, both industrial strain (Hu.Yo x Tukuba.Ne) and strains of transgenic silkworms larvae, reared on artificial diet. The larvae looked weakened and showed symptoms of diarrhea. From previous reports that Silkworm larvae infected with various pathogens, including bacteria, suffer from severe diseases (*6-9*), we hypothesized that the problems might be due to infection. First, we tested whether the weakened silkworms were infected with bacteria. Feces obtained from the weakened silkworm were suspended in sterilized physiological saline and spread on BHI agar plates. Homologous colonies formed on the BHI agar plate (Figure 1A). These isolated colonies were round-shape and appeared purple-coloured when viewed through a microscope after Gram staining (Figure 1B), suggesting that the suspected pathogen is Gram-positive cocci. Sequencing analysis of its 16S rRNA gene revealed that the sequenced gene of the isolated pathogen matched with that of a bacterium specie, *E. mundtii*. All together, these results suggest that the weakened silkworms were infected with *E. mundtii*.

3.2. *Median lethal dose (LD_{50}) determination via oral administration*

We next examined whether or not the isolated *E. mundtii* was pathogenic to silkworm. Results indicated that when full growth was injected into hemolymph of silkworms, the animals were killed within 72 h. The killing effects by *E. mundtii* was also observed when bacteria were administered by oral pathway through feeding with diet adsorbed with the bacterial full growth, though it took longer period unlike direct injection into hemolymph. We determined the median lethal dose, LD_{50} and found that the number of the bacteria needed to kill half population of tested silkworm larvae via oral administration was 1.04×10^6 CFUs after 6 days (Figure 2). Taken together, the above results demonstrated that *E. mundtii* was pathogenic to silkworm larvae, *Bombyx mori*.

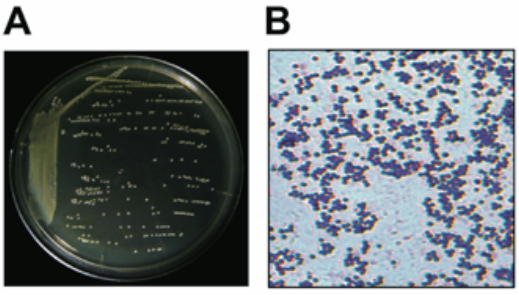

Figure 1. Isolation of colonies from feces of silkworm suffering from diarrhea. (A) Feces were obtained form silkworm with diarrhea, suspended in sterilized physiological saline and spread on BHI agar plates. The BHI plate was incubated at 30°C for 24 h. **(B)** Isolated single colony was suspended in sterilized physiological saline, Gram stained according to previous protocol (*15*) and observed with a microscope.

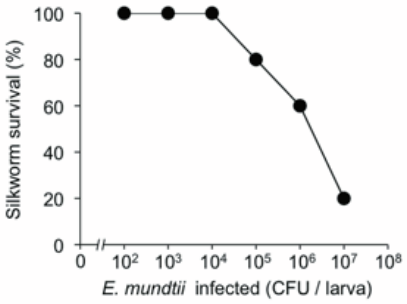

Figure 2. Lethal effects on silkworm by oral administration of *E. mundtii*. Various CFUs of *E. mundtii* were adsorbed onto artificial diet through the full growth culture. The infected diet was fed to larvae and incubated at 27°C. After 6 days, number of surviving silkworms ($n = 5$) was counted.

3.3. *Effects of mild E. mundtii infection on growth of silkworm larvae*

Next, we examined problems associated with infection of larvae with a smaller number of *E. mundtii*; much less than LD_{50}. Silkworm larvae were fed with artificial diet containing 1×10^5 CFUs/larva. The feeding was further continued for 5 days with non-infected artificial diet. Photo shot of the body sizes of silkworms was taken on the 6th day (Figure 3A). The weight of infected

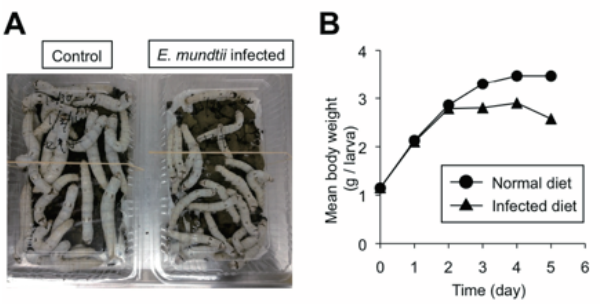

Figure 3. Inhibition of silkworm growth by oral administration of *E. mundtii*. (A) Silkworms were reared for 6 days at 27°C with normal diet (control) or diet containing 1×10^5 CFUs/larva of *E. mundtii* (*E. mundtii* infected). **(B)** Silkworms were reared at 27°C with normal diet or diet containing 1×10^5 CFUs/larva of *E. mundtii* (Infected diet). Body weight of the silkworms was monitored for 5 days and mean weight determined.

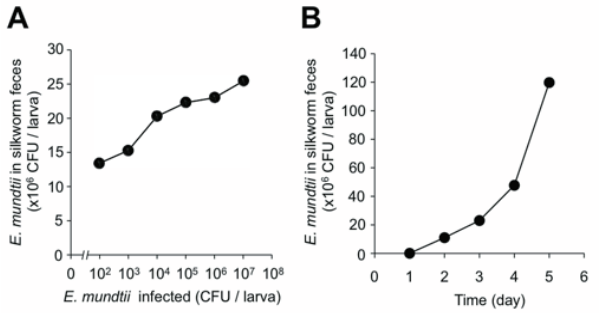

Figure 4. Proliferation of E. mundtii in silkworm larvae. (A) Various CFUs of *E. mundtii* (1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 CFUs/larva) were administered to silkworm via oral administration of diet infected with the full growth culture and bacterial population was monitored on day 3. **(B)** Larvae were infected (1×10^6 CFUs/larva) through infected diet administration and bacteria population monitored for 5 days. Five pieces of feces were obtained from each group (n = 5), suspended in sterilized physiological saline and spread on BHI agar plates. After incubation at 30°C for 24 h, the number of colonies that appeared on the plates was counted. Total *E. mundtii* CFUs present in feces were calculated.

larvae decreased in a time-dependent manner compared to non-infected (larvae) control, becoming very drastic beginning from 48 h post-infection (Figure 3B). Loss of appetite was also observed in the diseased animals. These results suggest that less severe *E. mundtii* infection of larvae results to anorexia and hampers the growth of the animals.

3.4. *E. mundtii proliferation in the gut of silkworm larvae*

Having observed the time-dependent detrimental effects of the infection, we next asked whether *E. mundtii* proliferated in the gut of silkworm larvae after oral ingestion. Silkworms were fed with artificial diet containing *E. mundtii*, and number of the bacteria in feces was determined by counting number of colonies after spreading of samples on agar plates followed by incubation. Results demonstrated that the bacterial population in the feces of larvae increased in both dose- and time-dependent manner (Figures 4A and 4B). These findings suggest that the bacteria proliferated in the gut (gastrointestinal tract) of silkworms, and that this could probably be responsible for its pathogenic effects in the host.

3.5. *Isolation of bacteria from artificial diet of silkworm*

Cappellozza *et al*. reported previously the infection of silkworm larvae reared on artificial diet by *E. mundtii* (*10*). Coupled with our observation, therefore, we suspected that the origin of *E. mundtii* was artificial diet. First, we attempted to isolate *E. mundtii* by incubation of pieces of the artificial diet in BHI medium. However, we could not obtain reproducible results of bacterial proliferation. Thus, we decided to examine the presence of *E. mundtii* on the powder of artificial diet before heat treatment. The manufacturer that provided the artificial diet (Silkmate 2S), which was heat-treated to cause agar polymerization, also provided the powder (Silkmate 2M) prior to heat treatment. Incubation of BHI agar plate that was streaked with suspension of the powder lead to appearance of bacteria colonies within 24 h. Sequencing and analysis of isolated bacterial 16S rRNA gene indicated that *E. mundtii* was present as a major population. Other colonies were identified by sequence of 16S rRNA as *Acinetobacter baumanni*, *Stenotrophonomonas matophilia*, *Pseudomonas geniculata*; and *Enterococcus gallinarum*, *casseliflavus*, and *faecalis* sub-specie. These findings suggest that the powder of the artificial diet prior to heat treatment contains viable *E. mundtii* cells and other bacteria.

3.6. *E. mundtii isolation from mulberry leaves*

Since the artificial diet contained powder made from mulberry leaves, we hypothesized that the origin of the infection was mulberry leaves. To test this, we examined the presence of the bacteria on the leaves. To establish a method for isolation of *E. mundtii* from mulberry leaves phylosphere, which may contain multiple microbial florae other than *E. mundtii*, we attempted to find growth conditions specific for *E. mundtii*. Since we have demonstrated that *E. mundtii* proliferated in intestine of silkworm under alkaline conditions, and corroborated by previous reports (*19,20*), we hypothesized that *E. mundtii* was resistant to alkaline conditions. To test this, we compared the growth of *E. mundtii* and *Staphylococcus aureus* or *Pseudomonas aeruginosa* (common environmental bacteria) on agar BHI plates with various concentrations of Sodium Hydroxide (NaOH). We found that among other specie of bacteria only *E. mundtii* could form colonies on plates containing 0.06 N NaOH, whereas the growth of *S. areus* and *P. aeruginosa* were terminated under the condition (Figure 5A). This information allowed

us to establish a selection method for *E. mundtii* from other bacteria. By using alkaline agar plates containing 0.06 N NaOH, we readily isolated bacterial colonies from mulberry leaves, which could grow under alkaline conditions (Figure 5B). The sequence of the 16S rRNA gene indicated that majority of bacteria on the alkaline plates were *E. mundtii*. From 1.0 g mulberry leaf sample, we isolated 2.6×10^5 bacteria colonies out of which 1×10^4 colonies were *E. mundtii*. These results suggest that the mulberry phyloplane contains *E. mundtii* among other epiphytes.

3.7. *Vancomycin treatment of diet decreased E. mundtii population in silkworm gut*

From above results, we hypothesized that small number of *E. mundtii* cells remains viable even after mild heat-treatment of artificial diet (employed by the manufacturer), and this could be responsible for the observed infection. Hence, we decided to establish a method to exclude the bacteria from silkworms. First, we attempted to exclude *E. mundtii* by treatment with antibiotics. To choose an appropriate antibiotic, we determined the MICs of ampicillin, vacomycin, kanamycin, chloramphenicol and tetracycline against *E. mundtii*. Among five antibiotics tested, vancomycin showed most potent activity for inhibiting the growth of *E. mundtii* as summarized in Table 1. These results suggest that the bacterium is highly susceptible to vancomycin treatment. Thus, we next examined whether the addition of vancomycin to diet would cause the disappearance of *E. mundtii* from intestine of silkworms infected with the bacteria. We added full growth of the bacteria to artificial diet followed by larvae feeding with the diet containing vancomycin (160 µg/g diet). Then, we examined the presence of the bacteria in the intestinal materials of the larvae. The results indicated that feeding of vancomycin-treated diet caused significant decrease ($p < 0.0001$) in the number of isolatable *E. mundtii* colonies, from average of 5.7×10^6 CFUs in the intestine of control to 3.6×10^3 CFUs in the treated animals 24 h post-administration of diet (Figure 6). From these results, we propose that vancomycin treatment could prevent *E. mundtii* infection in silkworm larvae possibly by inhibiting in the proliferation process *in-vivo*.

3.8. *Autoclaving and gamma ray irradiation of the artificial diet exclude E. mundtii*

High pressure-heat-based processing, and irradiation are two established methods of food sterilization that

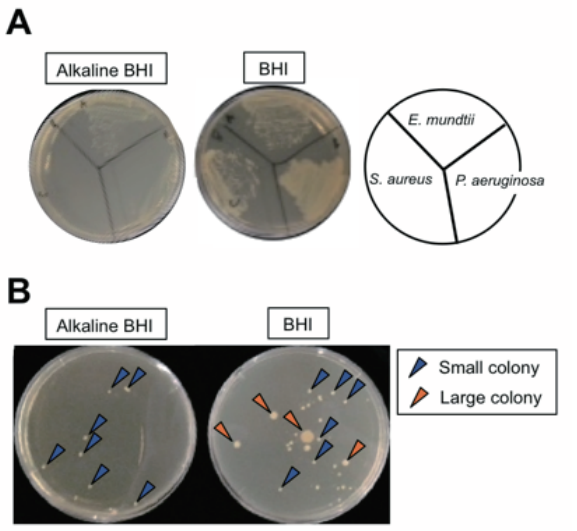

Figure 5. Isolation of *E. mundtii* from mulberry leaves using alkaline containing agar plate. (A) Single colonies of *E. mundtii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* were streaked on both BHI agar plate with 0.06 N NaOH (Alkaline BHI plate) and BHI agar plate (BHI). The plates were incubated at 30°C for 24 h. **(B)** Mulberry leaves were homogenized in sterilized physiological saline. The homogenized suspension was streaked on both BHI agar plate with 0.06 N NaOH (Alkaline BHI plate) and BHI agar plate (BHI). The plates were incubated at 30°C for 24 h.

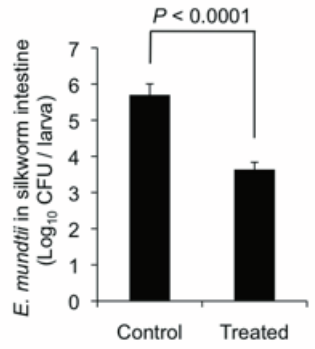

Figure 6. Vancomycin treatment inhibits proliferation of *E. mundtii* in silkworm. Larvae were fed with *E. mundtii* infected diet (overnight culture of E. mundtii, 5 µL/g diet) treated with vancomycin (160 µg/g diet) or without (control) for 24 h. Intestinal materials of silkworms were harvested and the number of CFUs determined. All data were analyzed using Graph-pad prism student *t*-test. Descriptive data ($n = 5$) were expressed as mean ± SEM. Statistical probability, $p < 0.0001$ was considered significant.

Table 1. MIC values of common antibiotics against *E. mundtii*

Strain	Minimum Inhibitory Concentration (µg/mL) of antibiotic				
	Ampicillin	Vancomycin	Chloramphenicol	Kanamycin	Tetracycline
E. mundtii	8	0.5	32	32	1

The minimum inhibitory concentration (µg/mL) was determined using broth dilution method of antimicrobial susceptibility testing as described previously (*11,12*).

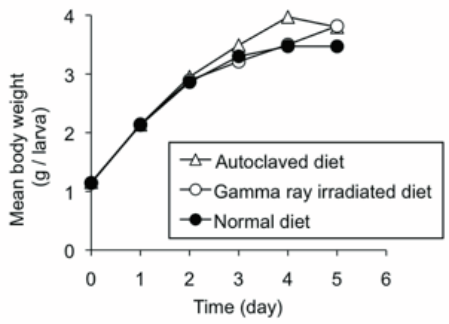

Figure 7. Autoclaving and gamma ray treatment of diet do not affect silkworm growth. Autoclaved diet (saline containing diet powder heat-treated to 121°C for 20 min) was prepared from Silkmate 2M. Gamma ray irradiated diet was prepared from artificial diet, Silkmate 2S. Silkworms (n = 10) were fed with normal diet, autoclaved diet or gamma ray irradiated diet at 27°C for 5 days. Body weight of the silkworms was monitored for 5 days and mean weight determined.

inactivate pathogenic bacteria employed in the food industry (*21,22*). Thus, we tested for the effects of these two methods; autoclaving and gamma ray treatment on the presence of the bacteria on the diet on one hand, and on the growth of fed animals on the other. When water suspension of diet powder (Silkmate 2M) was autoclaved at 121°C for 20 min, no bacteria colony was observed by examination of the materials spread on nutrient agar plates (data not shown), confirming our hypothesis that severe heat treatment of diet materials could kill the bacteria. The measurement of the weight of silkworms fed with the diet revealed that the autoclaving of the artificial diet did not affect the growth of the larvae (Figure 7). Gamma ray irradiation of the artificial diet also gave the result of complete loss of living bacteria. Silkworm larvae fed with the gamma ray irradiated diet also showed normal growth (Figure 7), compared to the normal diet fed animals. These findings suggest that autoclaving or gamma ray treatment of larvae diet powder or diet during preparation has no negative consequences on growth of the fed animals.

4. Discussion

In this current study, we observed the presence of *E. mundii* in intestine of diseased silkworm larvae, *Bombyx mori*, reared with artificial diet, which posed a serious challenge in screening of drug candidates. Our present results indicate that feeding of diet containing *E. mundtii* caused proliferation of the bacteria in silkworm gut. Infection of larvae by the bacteria led to weight loss, loss of appetite, and eventually caused death of larvae. Therefore, we concluded that *E. mundii* was pathogenic to silkworm larvae, *Bombyx mori*, hence poses a direct threat to experimental reproducibility during research; and should be excluded. We traced the origin of the bacteria and successfully isolated it from diet powder made from mulberry leaves. We also demonstrated that mulberry leaves contained E. mundii. Therefore, we concluded that the mulberry leaves was a source of infection. We tested the sensitivity of antibiotics against *E. mundtii*, and demonstrated that the bacteria showed high susceptibility to vancomycin treatment *in vitro*. *In vivo*, we found that when silkworm larvae were fed with vancomycin-treated infected diet (160 μg/g diet), the bacterial proliferation was significantly decreased (< 1%) in the intestine. Thus, we propose that vancomycin treatment is a possible method to exclude *E. mundii* infection from silkworm, although the complete loss of the bacteria was very difficult. Furthermore, we established that autoclaving or gamma ray irradiation of diet completely removed *E. mundtii* without affecting its nutrient content. However, when larvae were fed with mulberry leaves, *E. mundtii* proliferated in the gut although no apparent problem of growth of larvae was observed. We hypothesize that substances(s) present in mulberry leaves might regulate the pathogenicity of the bacteria in the body of the silkworm host. Another postulation is the possibility that co-existence of other bacteria in intestine contribute to avoid infection problems posed by *E. mundtii*. Further investigation is, therefore, needed to understand the mechanism by which silkworms escape from the *E. mundtii* infection problem even when fed with mulberry leaves infested with the pathogen.

In conclusion, this study identified the origin of *E. mundtii*, validated the bacteria as a pathogen of silkworm larvae and proposed methods for preventing the infection.

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