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Physiological comparison between mulberry (*Morus alba* L.) leaves diet and artificial diet on growth development as well as antibiotic therapeutic response of silkworms

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Abstract

Silkworm has been proposed recently as an animal model for the study of therapeutic effects of drug candidates. Little is known regarding the factors influencing the physiology of the silkworm, and this might become the major effect of the therapeutic result interpretation. In the present study, the effect of a diet of mulberry leaves as compared to an artificial diet on Thai silkworm growth development as well as the antibiotic therapeutic responses of bacterial infected silkworms is explored. The silkworms fed with mulberry leaves as well as those with artificial diets were weighed and the numbers of survival were counted. The mulberry leaves-fed silkworms (MFS) showed faster growth rate (0.2 g body weight day^{-1}) than the artificial diet-fed silkworms (AFS). The MFS exhibited bigger body size and greater body weight than the AFS. It was found that the median lethal dose (LD_{50}) of *Staphylococcus aureus* cells needed to kill 1-g body weight silkworms was 10^8 cells ml^{-1} for both MFS and AFS. However, the median effective dose (ED_{50}) of the antibiotics (ampicillin, ceftriaxone, cephalexin, and gentamicin) for AFS was two times higher than those for MFS. The results indicate that some elements in mulberry leaf affect the therapeutic effect of the antibiotics in the silkworm model. This study suggests that high attention on silkworm diet should be paid to the research relevant to drug therapeutic evaluation using silkworms as an animal model.

Keywords: therapeutic effect; antibiotics; silkworms; animal model; mulberry leaves; artificial diet.
Abbreviations: MFS_mulberry leaves-fed silkworms; AFS_artificial diet-fed silkworms; LD_{50}_median lethal dose; ED_{50}_median effective dose; MIC_minimum inhibitory concentration; MHB_Mueller-Hinton broth; NaCl_sodium chloride; ATCC_American type culture collection.

Introduction

Infectious diseases, particularly those caused by drug-resistant pathogenic bacteria, is one of the serious problems faced by under-developed and developing countries. Quest for new therapeutic agents that can effectively tackle this menace is urgently needed. Drug candidates screened through *in vitro* study have problems in pharmacokinetics since only a small portion of them show therapeutic effects in disease models with animals (Rotsami-Hodjegan and Tucker, 2007). However, the use of mammals, e.g., mice and rats, as animal models is not preferable because of the high costs and ethical issues involved (Baumans, 2004). Use of invertebrates like *Caenorhabditis elegans* and *Drosophila melanogaster* has been proposed (Lemaitre et al., 1996; Mahajan-Miklos et al., 1999; Seabra and Bhogal, 2009); however, these animals have limitations in that their bodies are very small and are not suitable for the injection of accurate volumes of samples into the body fluid. Silkworm has been demonstrated as an ideal invertebrate animal model system for basic research and screening of various potential drugs (Kaito et al., 2002; Hamamoto et al., 2004; Kaito and Sekimizu, 2007; Ishii et al., 2008; Hamamoto et al., 2009; Fujiyuki et al., 2010). The physiology of the animal is important for the evaluation of the experimentally obtained results. One possible cause for the physiological difference in animals is the variation in nutrients. Mulberry is the most important crop for sericulture. The leaves of mulberry have been used for centuries as the nutrient for silkworms. Extensive research on the various compounds and nutrients treated to mulberry has been carried out in several countries. Structural identification of dietary components essential for the feeding of silkworm larvae was investigated by Hamamura et al. (1962), and Ito and Mukaiyama (1964). More investigations on the relationship between dietary components and growth were conducted later (Ito, 1980; Telang et al., 2002; Hu et al., 2005). It was reported that good quality cocoons of silkworms could be obtained when silkworms were fed with nutritionally enriched mulberry leaves (Goudar and Kaliwal, 2000; Etebari and Matindoost, 2005). Jia and Yong (1988) reported that any fluoride from the ambient air was absorbed by the mulberry leaves, and that this had an effect on silkworm development. Leaves containing more than 80 ppm fluoride severely inhibited cocoon production. Khan et al. (2010) found that silkworm larvae, when reared on mulberry leaves treated with optimum doses of nitrogen, phosphorus, potassium and calcium solutions, significantly consumed more food, gained more larval weight and produced heavier cocoons as compared to those reared on untreated leaves. Noteworthily, the supplementation of the silkworm diet with selected amino acids at certain levels effectively improved silkworm growth and development (Radjabi, 2010). The studies on food preferences for larval growth promotion led to the development of an artificial diet. Today, the silkworm can be

Table 1. Minimum inhibitory concentration (MIC) and the ED_{50} of the antibiotics against *S. aureus* in the silkworm infection model as fed with different diets

Antibiotics	MIC (μg ml^{-1}broth)	ED_{50} (μg g^{-1}larva)		Ratios of ED_{50}/MIC	
		MFS[a]	AFS[b]	MFS[a]	AFS[b]
Ampicillin	0.05	0.03	0.06	0.6	1.2
Ceftriaxone	3.2	2.5	>16	0.8	>5
Cephalexin	1.6	0.8	1.6	0.5	1
Gentamicin	0.1	0.05	0.2	0.5	2

[a] — mulberry leaves-fed silkworms (MFS), [b] — artificial diet-fed silkworms (AFS)

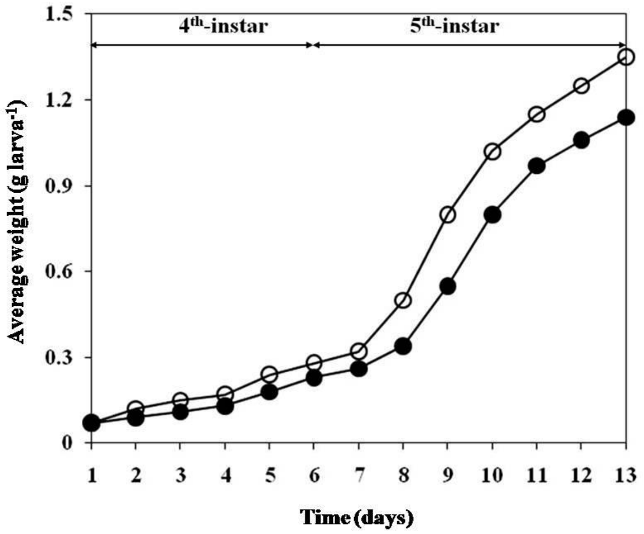

Fig 1. Average body weight of the mulberry leaves-fed silkworms (○) and artificial diet-fed silkworms (●), with diets given every day for 13 days since the 1st day of the 4th-instar larvae (n=100).

raised entirely on the artificial diet from the first to the last larval instars. Despite the abundance of nutritional studies on silkworm, the effect of diets on the therapeutic evaluation has not been reported. The present study explores the influence of mulberry leaves as a normal diet of silkworms as against the effect of the artificial diet on silkworm growth development, as well as the therapeutic response after antibiotic injection of the infected silkworms.

Results

Effect of diets on silkworm growth

Although the development of most insects is directly or indirectly controlled by hormones (Ismail and Dutta-Gupta, 1988), it can also be partly regulated by exogenous factors like food intake (Calvez, 1981). The results of two different diets fed to the 4th-instar silkworms demonstrated some significant differences in growth, as shown in Fig 1. It was noted that during the 4th-instar period, which covered the first 6 days, the gained body weight in the silkworms of both groups was slightly increased and not obviously different from each other. In the 5th-instar period, which started from day 7, the mulberry leaves-fed silkworms (MFS) increased in body weight more rapidly than the artificial diet-fed silkworms (AFS).

Effect of fasting on the survival of silkworms

A standard protocol for the evaluation of the therapeutic effect of antibiotics on silkworms includes subjecting the silkworms to fasting after the injection of the pathogenic bacteria (Hamamoto et al., 2004). Therefore, we examined the influence of the different diets on the survival of the silkworms after fasting. The survival rates and the variation in the body weights of the fasting and feeding control groups are shown in Fig 2. The fasting control group which was not given any diet along the period of experiment demonstrated that the body weight of the silkworms had begun to decrease after day 2, followed by a rapid increase in the death rate until day 5 when no silkworm was observed to survive (Fig 2A). The feeding control silkworms exhibited 100% survival, but the growth rates of the silkworms were different in the MFS and the AFS. On the last day of this period, the body weight of the MFS (Fig 2B) was obviously greater than that of the AFS (Fig 2C). Different feeding period of both diets to the silkworms showed an effect on silkworm survival (Fig 3). Feeding for the first 4 days with mulberry leaves or the artificial diet, resulted in 100% survival until day 6 (Fig 3A). The weights of the silkworms of both groups increased until day 5, and then decreased due to the fasting. Similar results were obtained when the silkworms were fed only for the first 3 days (Fig 3B). With both nutrients, the silkworms showed 100% survival until day 6. The body weight increased until day 4, and then decreased. The mulberry leaves diet and the artificial diet did not show any significantly different effects on the body weights. Feeding the silkworms only on the first 2 days caused a decrease in the number of the MFS that survived after day 5, whereas the number of the AFS that survived decreased after day 4 (Fig 3C). In addition, a half of the AFS was seen to be able to survive until day 7, while no silkworm was observed to have survived on day 7 in the MFS group ($p<0.1$). The MFS silkworms that survived were slightly bigger and heavier than the AFS. The result is similar to the silkworm groups fed only on the first day, followed by fasting (Fig 3D). The decreasing rate of the AFS was significantly lower than that of the MFS, indicating that the AFS could survive longer during starvation than the MFS.

Table 2. Concentrations (μg ml^{-1}) of the antibiotics used for injection to the silkworms.

Ampicillin	Ceftriaxone	Cephalexin	Gentamicin
0.05	3.2	1.6	0.1
0.25	16	8	0.5
0.5	32	16	1
1	64	32	2
2.5	160	80	5
5	320	160	10

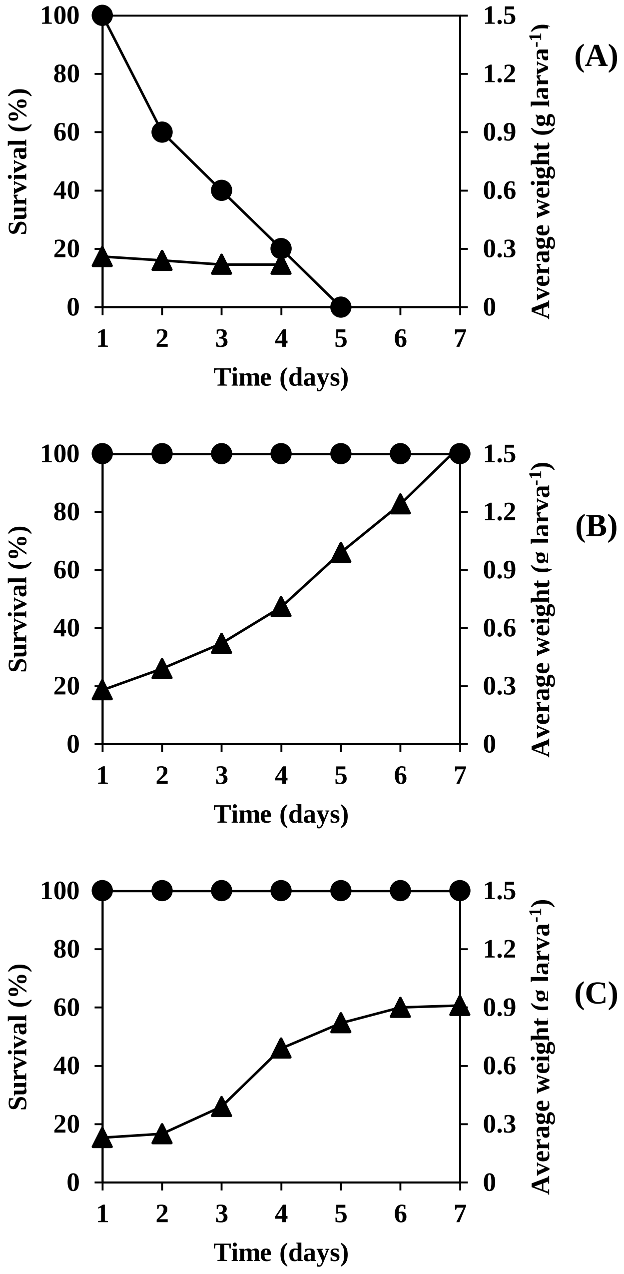

Fig 2. Survival (●) and body weight (▲) of the silkworms in the fasting control group (A), the feeding control groups fed with mulberry leaves (B), and the feeding control groups fed with artificial diet (C). All silkworms were kept at 30°C. The survived silkworms were counted and their body weights were measured.

Effect of diets on killing of silkworms by bacteria

Staphylococcus aureus ATCC 25923 was used as the pathogenic bacteria for killing the silkworms. It was found that number of the surviving silkworms decreased with an increase in bacterial concentration and the time after injection. There was no significant difference in the degree of survival between the AFS and the MFS after the bacterial injection. In further experiments, silkworms of different body weights were investigated: the 5^{th}-instar MFS of 1-g body weight (lighter) were compared with those of 1.5-g body weight (heavier). The results demonstrated that the 10^6 and 10^7 cells ml^{-1} bacterial suspensions could not kill the heavier silkworms, whereas the 10^7 and 10^8 cells ml^{-1} bacterial suspensions had distinct killing effect on the lighter silkworms. Half of the lighter silkworms were killed on day 3 by the injection of 10^8 cells ml^{-1} bacterial suspension, whereas all of the heavier silkworms survived. The 10^9 cells ml^{-1} bacterial suspension killed the entire lighter and the heavier silkworms on day 1 and day 4 after injections, respectively.

Impact of diets on therapeutic effect of antibiotics in infected silkworms

The results as shown in Table 1 indicate that MIC values of the tested antibiotics against *S. aureus* were ranged from 0.05 – 3.2 μg ml^{-1}. Ampicillin displayed the strongest inhibition whereas ceftriaxone showed the lowest inhibition with the MIC of 0.05 and 3.2 μg ml^{-1}, respectively. All the silkworms injected with 100-fold MIC of each drug, without bacteria, showed 100% survival, indicating that the dose of antibiotics used was nontoxic to the animals. Meanwhile, all the silkworms injected with *S. aureus* were killed, thereby indicating the killing potential of the bacterial dose as regards the tested silkworms. The antibiotics concentrations ranged from MIC to 100-fold MIC injected to the silkworms showed different survival effect to the animals as shown in Fig 4. The results demonstrate that the antibiotics could increase the survival of the infected silkworms and that the effect was dose-dependent. Both mulberry leaves diet and artificial diet showed distinct results on the therapeutic effects of antibiotics. The quantity of antibiotics that effected 50% survival of the silkworms (ED_{50}) was calculated from the survival curves. It was found that the ED_{50} values of ampicillin, ceftriaxone, cephalexin and gentamicin for the AFS were more than two times those for the MFS. As the MFS and the AFS groups showed no significant difference regarding resistance to the bacteria (mentioned above), the differences in the ED_{50} values of the antibiotics tested by the different diet-fed silkworms need to be explained via other effects.

Discussion

In this study, a diet of mulberry leaves showed many different effects on the silkworms as compared to an artificial diet. The first experiment demonstrates the effects on the silkworm growth development and on the starving resistance. The silkworms fed with mulberry leaves showed faster growth than those fed with the artificial diet. Mulberry leaves have high (75 to 85%) *in vivo* digestible dried matters (Kandylis et al., 2009). The leaves also contain sugars, proteins, fats, moisture, fibres and organic acids.

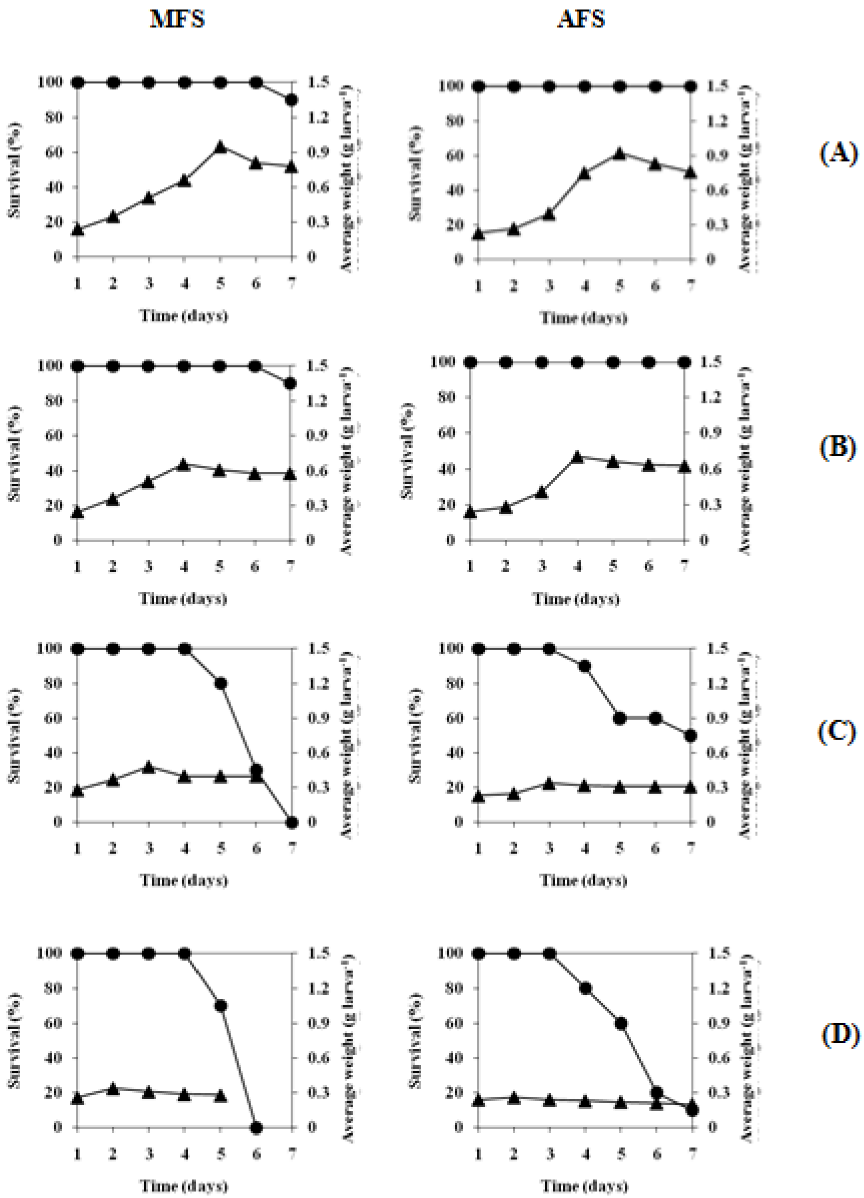

Fig 3. Survival (●) and body weight (▲) of the fifth-instar silkworms fed with mulberry leaves (left) and with artificial diet (right) for the first 4 days (A), the first 3 days (B), the first 2 days (C) and the first day (D). All the silkworms were kept at 30ºC. The silkworms that survived were counted and their body weights measured. The silkworms that were fed with mulberry leaves and those that were given an artificial diet for the first 2 days (C) showed significant differences as p<0.1 by log-rank test.

The results of this experiment indicated that mulberry leaves contain more essential compounds suitable for the growth of silkworm as compared to an artificial diet. Bosquet (1983) reported that food intake is not only related to the growth of the animal, but also acts as a developmental signal. From the results of the present experiment, we infer that some compounds in mulberry leaves might act as developmental signals for the silkworms. During starvation, the survival numbers of those silkworms fed with artificial diet were greater than those of the ones fed with mulberry leaves. Silkworms store energy as glycogen, lipid and storage protein (Janarthanan et al., 1999; Nagata and Kobayashi, 1990; Horie and Inokuchi, 1978), which are obtained by conversion from food ingredients. The ingredients of mulberry leaves and those of the artificial diet are different. The artificial diet contains soybean protein as the amino acid source (Ito et al., 1975; Escaffre and Kaushik, 1995), glucose as the sugar source (Kim and Jang, 2011) and soybean oil as the fatty acid source (Kandylis et al., 2009). These differences might explain the higher growth rate of the AFS in comparison with the MFS. The ED_{50}/MIC ratio is well correlated to the pharmacokinetic feature of antibiotics. The ratio values of the silkworms fed with the artificial diet were more than two times greater than those fed with the mulberry leaves, suggesting that the ingredients in mulberry leaves affect the pharmacokinetics of the antibiotics in silkworms. Sun et al. (2011) reported that mulberry leaves contain various compounds that can inhibit the activity of cytochrome P450,

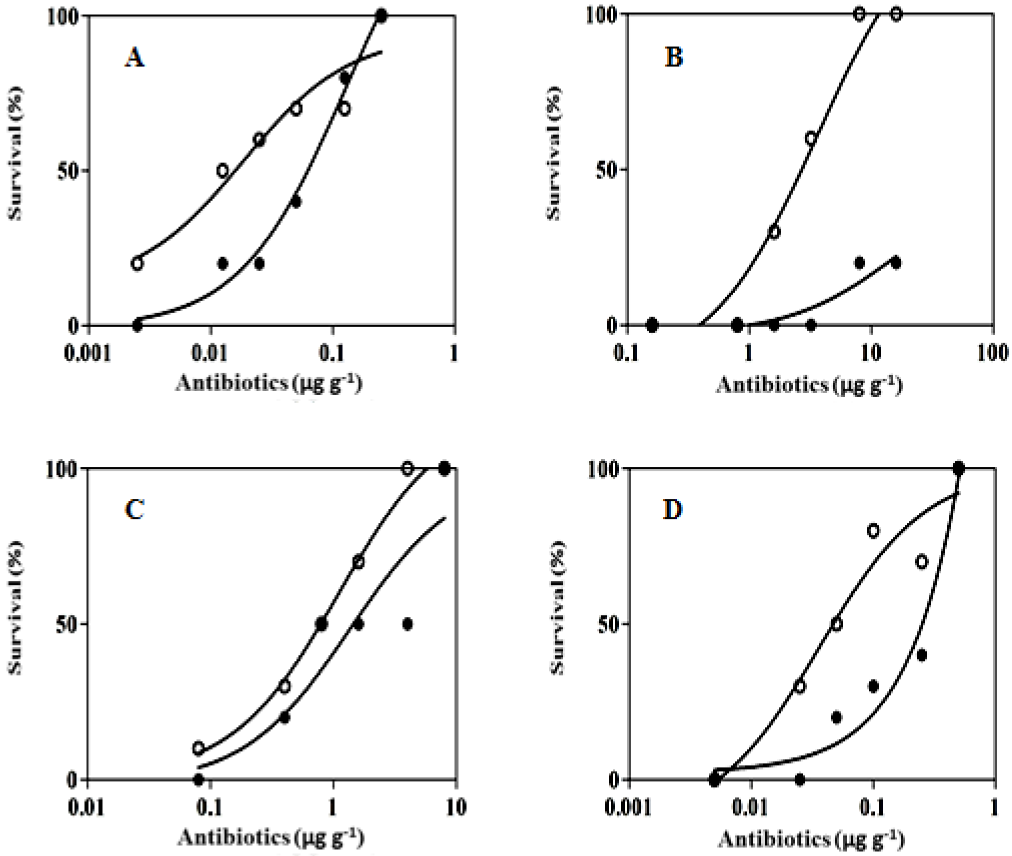

Fig 4. Survival of infected MFS (○) and AFS (●) treated with ampicillin (A), ceftriaxone (B), cephalexin (C) and gentamicin (D). After injection, all the silkworms were kept at 30°C for 2 days. The silkworms that survived were counted.

a major enzyme involved in drug metabolism. It is possible that the degradation of antibiotics by this enzyme in the MFS silkworm body was inhibited by the compounds in the mulberry leaves. This might have led to the sustained high drug concentration in the haemolymph of the silkworms and supported the survival of the silkworms against the injected bacteria, resulting in the reduction of their corresponding ED_{50} values. Zhou et al. (2008) reported that different diets could alter the expression of proteins in relation to the immune system, digestion and absorption of nutrients, and energy metabolism in silkworms. The report suggests that the different diets, mulberry leaves and artificial diet, may affect the immunological resistance of silkworms to bacterial infection. Further studies of the chemical compounds in mulberry leaves responsible for lowering the effect of the ED_{50} of antibiotics are needed to establish Thai silkworms as a model animal for the evaluation of the therapeutic effects of antibiotics.

Materials and methods

Nutrients and chemicals

Pesticide-free fresh mulberry leaves were collected from the mulberry trees grown in Queen Sirikit Sericulture Center (Chiang Mai, Thailand). Antibiotic-free artificial diet was provided by Laboratory of Microbiology, the University of Tokyo (Tokyo, Japan). Ampicillin, ceftriaxone and gentamicin were from BIC Chemicals (Nonthaburi, Thailand). Cephalexin was kindly gifted by Siam Pharmaceuticals (Bangkok, Thailand). Mueller-Hinton Broth (MHB) was from Merck, Darmstadt, Germany. All the other chemicals and reagents used in this study were of reagent grade.

Silkworms and microorganisms

The silkworms of Thai breeding (*Bombyx mori* L. var. *nangtui*) at the state of the fourth-instar larvae were kindly provided by Queen Sirikit Sericulture Center (Chiang Mai, Thailand). The silkworms obtained were reared on mulberry leaves or artificial diet in plastic packages with paper towels, and kept in the safety cabinet at 30ºC until further experiments. The paper towels were refreshed and the plastic packages were cleaned every day. Standard strain of *Staphylococcus aureus* ATCC 25923 was obtained from the Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand. The bacterial strain sub-cultured in the MHB was incubated overnight at 37°C. The bacterial suspension was prepared by diluting the cells with 0.9% sodium chloride (NaCl) sterile solution to a desired concentration. Freshly prepared suspension was used throughout the test.

Effect of diets on silkworm growth

The 4^{th}-instar silkworms with the same body weight were divided into two groups, each group containing 100 silkworms. One group was fed with mulberry leaves whereas the other was fed with the artificial diet. All the tested silkworms were kept in a plastic package laid with a clean paper towel, and cultured at 30°C. The mulberry leaves or the artificial diet was given every day for 13 days. The size and morphology of the silkworms were observed by visualization, and the body weight of the silkworms was determined using an analytical balance during the period of study.

Effect of fasting on the survival of silkworms

The 5^{th}-instar silkworms with the same body weight were taken from the cabinet and divided into two batches; one batch was treated to mulberry leaves whereas the other was treated to the artificial diet. Each batch was divided into six groups, with each group consisting of 10 silkworms. The first group, which served as a fasting control, was made to fast throughout, right from the 1^{st} day. The 2^{nd} group was fed only on the 1^{st} day, and then made to fast. The 3^{rd} group was fed on the 1^{st} and the 2^{nd} days, and then made to fast. The 4^{th} group was fed on the 1^{st}, 2^{nd} and 3^{rd} days, and then made to fast. The 5^{th} group was fed on the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} days, and then made to fast. The last group, which served as a feeding control, was fed every day. All the silkworms were kept at 30°C. The silkworms that survived were counted and their body weights measured. The size and morphology of these silkworms that survived were observed visually. The dead silkworms were separated from the group. The paper towels laid in the plastic packages were refreshed every day.

Effect of diets on killing of silkworms by bacteria

The 5^{th}-instar 1-g body weight silkworms were divided into 2 batches according to the two different nutrients. Each batch was divided into 6 groups; 2 control groups and 4 test groups. The test groups were injected with 0.05 ml of 10^6, 10^7, 10^8 and 10^9 cells ml^{-1} bacterial suspensions, respectively. The positive control group was injected with 0.05 ml of 0.9% NaCl without bacteria, and no treatment was done for the negative control group. The injection was performed by using a 27-gauge needle pushed into the haemolymph through the dorsal surface of the silkworm. All the silkworms were kept at 30°C. The silkworms that survived were counted. The size and morphology of the silkworms that survived were observed visually. In order to investigate the effect on body weight, the MFS with body weights of 1.5-g and 1-g were compared.

Determination of MIC of antibiotics

The determination of MIC, the lowest concentration of the antibiotics capable of inhibiting bacterial growth completely (Vuotto et al., 2000), was performed by using a broth dilution method previously described by Hamamoto et al. (2004). The turbidity indicates *S. aureus* survival was visually investigated in two-fold serial dilutions of tested antibiotics in the MHB. Cultures were incubated at 37°C for 24 h. The determination of MIC was in accordance with the National Committee for Clinical Laboratory Standards (2000)**.**

Impact of diets on therapeutic effect of antibiotics in infected silkworms

In this experiment, the 4^{th}-day fifth-instar silkworms were divided into 2 batches according to the 2 different diets. Each silkworm batch was divided into 30 groups, with each group consisting of 10 silkworms. Two groups were served for the controls. The positive control group was injected with 0.05 ml of the highest concentration of each antibiotic solution without the bacteria. The negative control group was injected with the 10^8 cells ml^{-1} bacterial suspension without the antibiotics. The 24 tested silkworm groups were injected with 0.05 ml of 10^8 cells ml^{-1} of *S. aureus* dispersion, immediately followed by 0.05 ml each of the respective antibiotics with different concentration as shown in Table 2. The bacterial suspension or drug solution was injected into the haemolymph through the dorsal surface of the silkworm body by using a 27-gauge needle. After injection, all the silkworms were kept at 30°C for 2 days. The silkworms that survived were counted. The size and morphology of the silkworms that survived were observed visually. The ED_{50} was calculated as the quantity of antibiotics required for 50% survival under conditions in which more than 90% of the silkworms were killed by the *S. aureus* infection.

Statistical analysis

Log-rank test was performed by Prism5 for Mac OS X (GraphPad Software, Inc.).

Conclusion

The feeding of different diets, mulberry leaves and artificial diet, affect both growth of silkworms and the therapeutic effect of antibiotics on silkworms. The results of this study suggest that some elements in mulberry leaf affect the therapeutic effect of the antibiotics in the silkworm model.

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