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# Effect of Lactic Acid Supplementation on the Growth and Reproduction of *Bombyx mori* (Lepidopteria: Bombycidae)

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## Abstract

Lactic acid is widely used in the food, drugs, cosmetics, and other industries to maintain the microbial stability of low-pH products. However, it is unclear whether lactic acid can affect silkworm (*Bombyx mori*) growth and reproduction. This study investigated the effects of lactic acid on the growth and reproduction of the silkworm. We analyzed the growth, cocoon quality, and reproductive performance of fifth instar larvae fed on mulberry leaves saturated with different concentrations (0.01, 0.1, 1, and 10%) of lactic acid and the control. Results showed that 0.01, 0.1, and 1% lactic acid supplementation positively affects growth and female cocoon quality, with increased larval weight and female cocoon shell weight compared to the control group. In contrast, 10% lactic acid was toxic to the larvae and significantly decreased growth, leading to larval death. Our study provides a basic reference for the optimal amount of preservatives. In addition, this study can be a desirable intervention for sericulturists and can play an important role in getting high return from silkworm-rearing activities.

**Key words:** lactic acid, *Bombyx mori*, growth, cocoon quality, reproduction

As a Lepidoptera model insect, the silkworm (*Bombyx mori*) has become an excellent material for classical genetics because of its abundant germplasm resources. It is also considered an important material for physiological, biochemical, and developmental biology research because of its short life cycle, convenient feeding, and complete metamorphosis (Jiang et al. 2020a). One of the most important characteristics of silkworm is its ability to convert plant protein into silk protein (Ude et al. 2014). Silk proteins have wide range of applications in medical sciences and industry (Khyade and Yamanaka 2018, Ageitos et al. 2019). Mulberry (*Movus alba*) leaves are the only natural food of silkworms. Larval growth and production of high-quality silk by silkworms depend largely on the various nutrients and quality of mulberry leaves offered (Smitha and Rao 2010). China is the largest silk producer in the world and has a great demand of mulberry gardens. However, with the development of the economy and society, severe environmental pollution, and higher labor costs, the area available for mulberry garden has been gradually reducing and silkworms are being increasingly reared on an artificial diet.

The development of artificial diets is an important direction in the sericulture industry. It is of necessary to promote the large-scale and labor-saving breeding of silkworms, in order to modernize the sericulture industry. The artificial diet of silkworms generally comprises mulberry leaf powder, defatted soybean powder, corn powder, mulberry green branch powder, cellulose, molding agents, vitamins, preservatives, and inorganic salt, which can adapt to the feeding characteristics of silkworms and meet the nutritional needs of silkworms. Currently, most of the improvements are aimed at improving the nutritional composition of the artificial diet (Saviane et al. 2014, Shifa 2016, Bhatti et al. 2019). Shifa (2016) found that supplementation of 10% Zea mays flour significantly increases silkworm larval weight, cocoon weight, and cocoon shell weight compared to controls (Shifa 2016). Additionally, Bhatti et al. (2019) found that adding 2% aqueous honey enhances larval growth and the silk cocoon yield (Bhatti et al. 2019).

Another important aspect in artificial diets is the preservatives added to inactivate or inhibit the growth of spoilage microorganisms and prolong the shelf life of food. Currently, the preservatives used in artificial diet of silkworms are sorbic acid, propionic acid, or compound substances (Cui et al. 2016, Yang et al. 2016). The research on artificial diet preservatives has not made substantial progress, and most of the research focuses on the development of chemical preservatives, although their effects on the growth and development of silkworms are still unclear. Organic acids are naturally occurring compounds found in many foods of plant origin, as well as being produced during fermentation of foods (Dang et al. 2009, Gurtler and Mai 2014, Elahi and Fujikawa 2019). Among them, lactic acid is widely used in the food, drug, cosmetics, and other industries to maintain the microbial stability of low-pH products (Beales 2004, Dang et al. 2009). However, it is unclear whether lactic acid can affect silkworm growth and reproduction.

This study investigated the effects of lactic acid on the growth and reproduction of silkworms. To determine the optimal concentration of lactic acid in the artificial diet, we treated mulberry leaves with different concentrations of lactic acid and measured the fifth instar larval weight; life span of the fifth instar larvae and pupae; cocoon weight, cocoon shell weight, pupal weight; female fecundity, and fertilization rate of silkworms. These results offer a safety assessment of whether lactic acid can be included in the artificial diet for silkworms and provide a basic reference for the optimal amount of preservatives.

## Materials and Methods

### *Bombyx mori* Strains and Rearing

We obtained silkworm larvae (Chufeng×Hanyun strains) from Industrial Crops Institute, Hubei Academy of Agricultural Sciences, Wuhan, China. Fifth instar larvae were kept in rearing boxes (40.5 × 19.5 × 9.5 cm) and fed fresh mulberry leaves at 25–28°C in a 12:12 (L:D) h environment with a relative humidity of 70–75%.

### Lactic Acid Supplementation

We diluted lactic acid with water to prepare different concentration (0.01, 0.1, 1, and 10%) solutions. Then, 5 liters of lactic acid of each concentration was placed in different clean glass containers. Fresh and healthy mulberry leaves were collected from a mulberry garden and completely soaked in the containers of lactic acid for 30 s to 1 min so that the leaves would be evenly coated with lactic acid. The leaves were then dried naturally at room temperature for 1–2 h before being presented to larvae as a food. The control group fed with mulberry leaves with ultrapure water, as previously described (Jiang et al. 2020b).

The larvae on the first day after the forth molt (fifth instar larvae) were used for all feeding experiments. We randomly divided the healthy and active fifth instar larvae with same size and age into five groups ($n$ = 180 larvae in per group). Three biological replicates with 60 larvae were performed for each group. The larvae were fed three times a day at 0600, 1400, and 2200 hours until they reached full maturity and began to form cocoons. The larvae in each group were offered approximately 150 g of mulberry leaves around on days 1 and 2, 180 g on days 4 and 5, and 150 g on day 6 to ensure adequate diet. The rearing boxes were regularly cleaned out by removing leftover leaves, feces, and dead larvae before next feeding to prevent diseases. We weighed all live larvae from each group every day before the first feeding. The growth status of larvae and the number of dead individuals in each group were recorded at the time of feeding.

### *Bombyx mori* Fifth Instar Larval Weight

We statistically analyzed the fifth instar larval weight, as previously described by (Jiang et al. 2020a). Briefly, we weighed (in grams) 40 larvae from each group every day until > 50% of the larvae had pupated. Finally, we calculated the daily weight gain average and the total weight of 6 d.

### *Bombyx mori* Pupal, Cocoon, and Cocoon Shell Weight

On the day 7 of the fifth instar, we manually collected mature larvae and transferred to mountages for cocooning. After a week, we recorded the number of dead individuals from each group. Next, we carefully opened the whole cocoons with a sharp blade to observe pupal characters. We identified the sex based on the taxonomic characters, which females have a line at the posterior end of the abdomen and males have a spot. We randomly selected 10 females and 10 males from each group to weigh the cocoon, pupae, and cocoon shells (in grams) using analytical balance. We also calculated the mean weight/shell (in grams).

### *Bombyx mori* Cocoon Shell Rate

We calculated the cocoon shell rate for each group, as previously described by (Jiang et al. 2020a):

$$\text{Cocoon shell rate} = (\text{Cocoon shellweight}/\text{Cocoon weight}) \times 100\%$$

#### Fecundity and Fertilization Ratio

On day 7 of pupa stage, we carefully opened the cocoons using a sharp blade and separated the male and female pupae. Once the pupae had emerged as a moth, 1-d-old males and females were subjected to a 1:1 mating in each group. Males and females were placed in a mating chamber for 6 h followed by manual decoupling. After mating, each female moth was kept for oviposition individually for 12 h. After egg-laying, we calculated the number of eggs and unfertilized eggs laid per female.

### Statistical Analysis

For all experiments, SPSS 19.0 was used for statistical analysis. A two-tailed Student's $t$-test was used to compare the control group with other groups. All data were represented as the means ± SD and graphed using GraphPad Prism 8.

## Results

### Effects of Lactic Acid on *B. mori* Fifth Instar Larval Weight

The larval stage of silkworms is an important period of growth and development, and the larval weight can reflect larval development. Therefore, in this study, from day 1 of the fifth instar larvae, we counted the number of dead larvae daily, measured the daily weight of fifth instar larval weight after supplementation with different concentrations of lactic acid (Fig. 1), and calculated the average daily weight gain (Fig. 2). There were no significant differences between the treated and control groups in terms of fifth larvae mortality rate (0.67% ± 0.01, 0.56% ± 0.01, 1.7% ± 0.02, 3.90% ± 0.02, 1.10% ± 0.02 for 0.01, 0.1, 1, and 10% concentrations and the control group, respectively). Before treatment, we observed insignificant differences in the average weights of the experimental and control groups (Fig. 1). From day 1 to day 5, 0.01, 0.1, and 1% lactic acid supplementation increased the larval weight. Lactic acid supplementation by 1% increased the larval weight every day compared to the controls. In addition, on day 5, the experimental group fed with mulberry leaves supplemented with 0.1% lactic acid had the highest weight compared to the other four groups (Fig. 1). On day 6, the weight of some groups decreased because some silkworms had already matured. However, the experimental group fed mulberry leaves supplemented with 10% lactic acid showed a significantly lower weight compared to control group every day (Fig.1).

Supplementation with 0.01 and 0.1% lactic acid significantly increased the average larval daily weight gain on day 1 (Fig. 2a), while 0.01 and 1% lactic acid supplementation significantly increased larval daily weight gain on day 2 (Fig. 2b). Although 10%

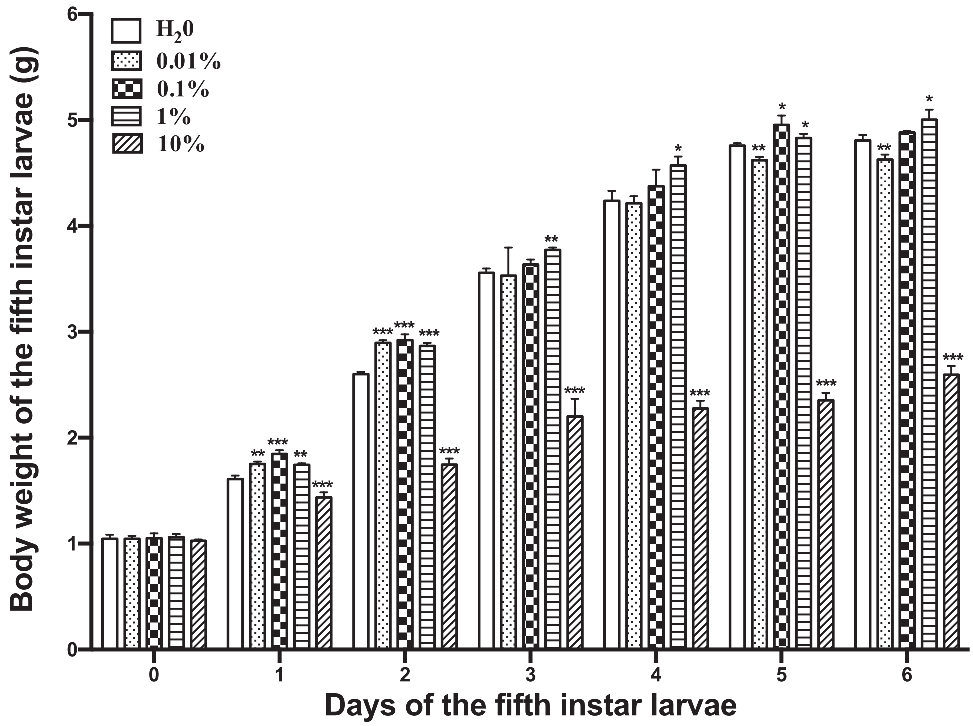


**Fig. 1.** Body weight of the fifth instar larvae daily. Data were presented by means ± SDs ($n = 3$). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

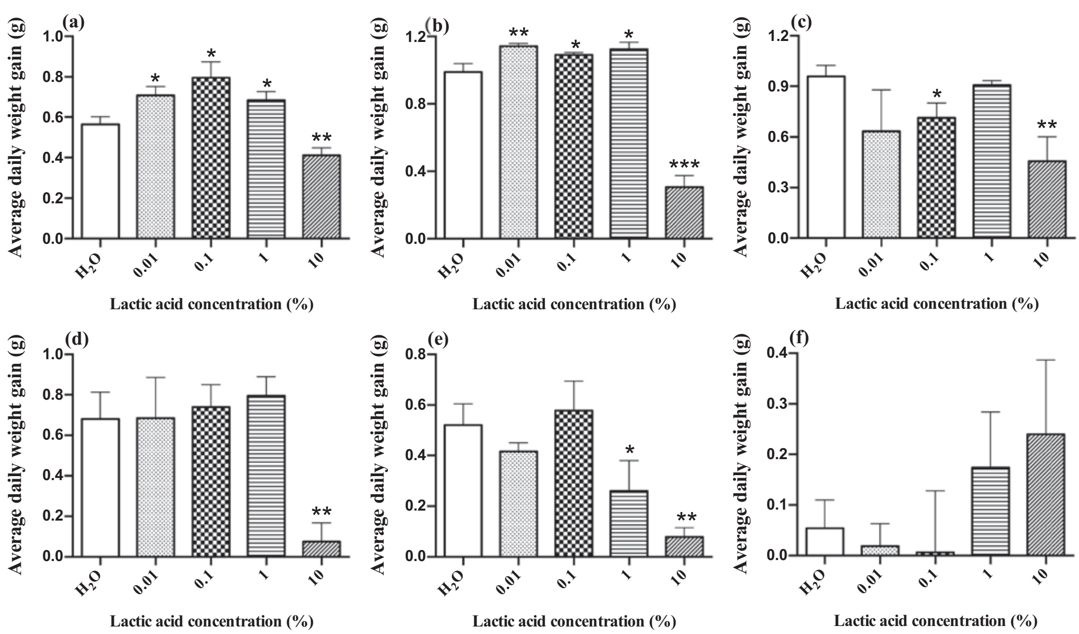


**Fig. 2.** Effect of lactic acid on average daily weight gain on different days of fifth instar larvae. (a) first day in the fifth instar; (b) second day in the fifth instar; (c) third day in the fifth instar; (d) fourth day in the fifth instar; (e) fifth day in the fifth instar; (f) sixth day in the fifth instar. Data were presented by means ± SDs ($n = 3$). $*P < 0.05$; $**P < 0.01$.

lactic acid supplementation increased larvae weight, the average daily weight gain was the lowest compared to other groups except day 6. In addition, larvae fed mulberry leaves supplemented with 10% lactic acid remained larval statues and could not spin a cocoon.

## Effect of Lactic Acid on the Larval and Pupal Life Span of *B. mori*

In this study, we investigated the effect of continuous feeding on the life span of fifth instar larvae and pupae (Fig. 3). After 10% lactic acid supplementation, the larvae could not finish cocooning and

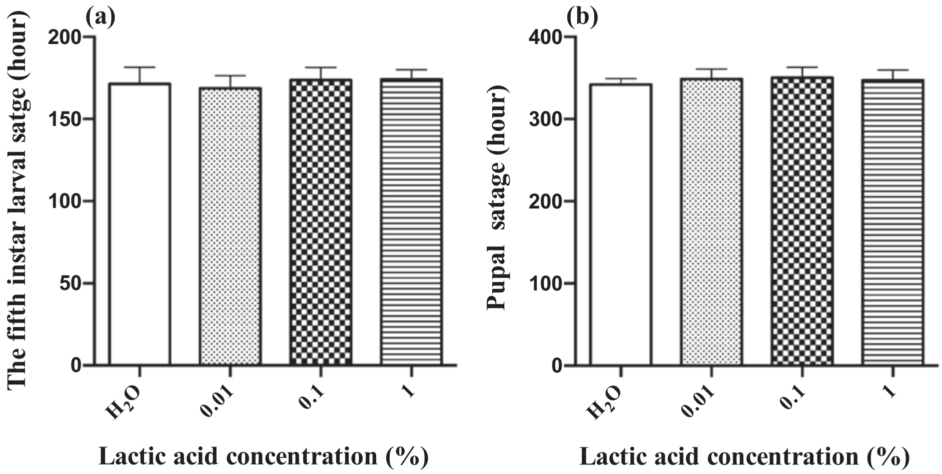


**Fig. 3.** Effect of lactic acid on the life span of Bombyx *mori*. (a) fifth larval stage; (b) pupal stage. Data were presented by means ± SDs ($n$ = 3).

pupation, so we had no data of the highest concentration. There was no significant difference between the experimental and the control group in terms of the fifth larval and pupal life span, indicating that lactic acid does not regulate the larval and pupal development time.

### Effects of Lactic Acid On *B. mori* Cocoon Production

In the sericulture industry, the cocoon shell weight and cocoon shell rate can reflect the cocoon quality. Therefore, after lactic acid supplementation, we investigated cocoon production on day 7 after cocooning. Lactic acid had no effect on the cocoon weight (Fig. 4a and b) and male pupal weight (Fig. 4d). Supplementation with 0.01% lactic acid decreased the female pupal weight, while other concentrations had no effect on pupal weight compared to the control group (Fig. 4c).

Lactic acid had no effect on cocoon shell weight and rate in males (Fig. 4f and h). The cocoon shell weight and rate of females in all experimental groups were significantly higher compared to those in the control group. These results showed that an appropriate concentration of lactic acid could increase female cocoon production.

### Effects of Lactic Acid on the Fecundity and Fertility of *B. mori*

Fecundity and fertility are the two main factors in *B. mori* reproduction. We calculated the fertilization rate by counting and recording fertilized and unfertilized eggs, which demonstrated the effect of lactic acid on the fecundity and fertility rate. There was no difference in the number of eggs laid in experimental groups control groups (Fig. 5a). Similarly, there was no difference in the fertilization rate between experimental and control groups (Fig. 5b). These results showed that lactic acid does not regulate female fecundity and fertility.

## Discussion

In the present study, we investigated the impact of lactic acid on the growth, cocoon production, and reproduction of *B. mori*. We found that supplementation with a suitable concentration (0.01–1%) of lactic acid somehow promoted larval growth. These results were in agreement with previous studies that showed lactic acid bacteria could promote the growth and development of fruit flies and could enhance immunity in both fruit flies and silkworms (Nishida et al. 2016, Li et al. 2017). One potential reason for our results is that lactic acid inhibits the growth of spoilage microorganisms and prolongs the shelf life of mulberry leaves that grow in the wild. Another reason could be that lactic acid lowers the pH of the hosts' intestinal tract and inhibits the growth and reproduction of pathogenic microorganisms while maintaining intestinal environment homeostasis, thus promoting silkworm larval growth.

In our study, a low concentration of lactic acid increases larval growth, whereas a high concentration of lactic acid inhibits larval feeding and decreases larval growth. Steib et al. (2001) reported that yellow fever mosquitoes show a preference for food supplemented with lactic acid in human odor samples (Steib et al. 2001). It is speculated that a low concentration (0.01, 0.1, and 1%) of lactic acid could enhance larval growth by increasing the appeal of mulberry leaves to silkworm larvae, whereas a high concentration of lactic acid (10%) might reduce leaf quality, resulting in the larvae showing some inclination to mulberry leaves.

After reaching full maturity, the silkworm larvae stop feeding and start cocooning. In our study, the cocoon yield increased only in females, indicating that lactic acid could enhance silk gland development and promote silk protein production only in females. This result needs to be investigated further by weighing silk glands and detecting silk protein expression.

An artificial diet preservative must effectively inhibit microbial production that causes food deterioration, but must have no adverse effects on the feeding and growth. In this study, adding a low concentration of lactic acid enhanced silkworm growth and female cocoon production, but did not affect larval and pupal life span and female fecundity and fertility. Therefore, as a weak organic acid, lactic acid can be added as a preservative to the artificial diets.

For a long time, sericulturists have been trying to increase the yield of silk cocoons with low inputs. Artificial diets could greatly reduce the labor and management costs of mulberry gardens. The labor productivity of sericulture has increased by 3 times, the scale of sericulture per household has increased by 2.5 times, and the labor time required 1kg cocoon has been reduced from 2.13 to 0.6 h by the introduction of artificial diets (Huang et al. 2013). Therefore, artificial diets are comparatively cost effective and easily manageable. Adding a suitable amount of lactic acid to the diet of silkworms could enhance cocoon production in females. Therefore, lactic acid

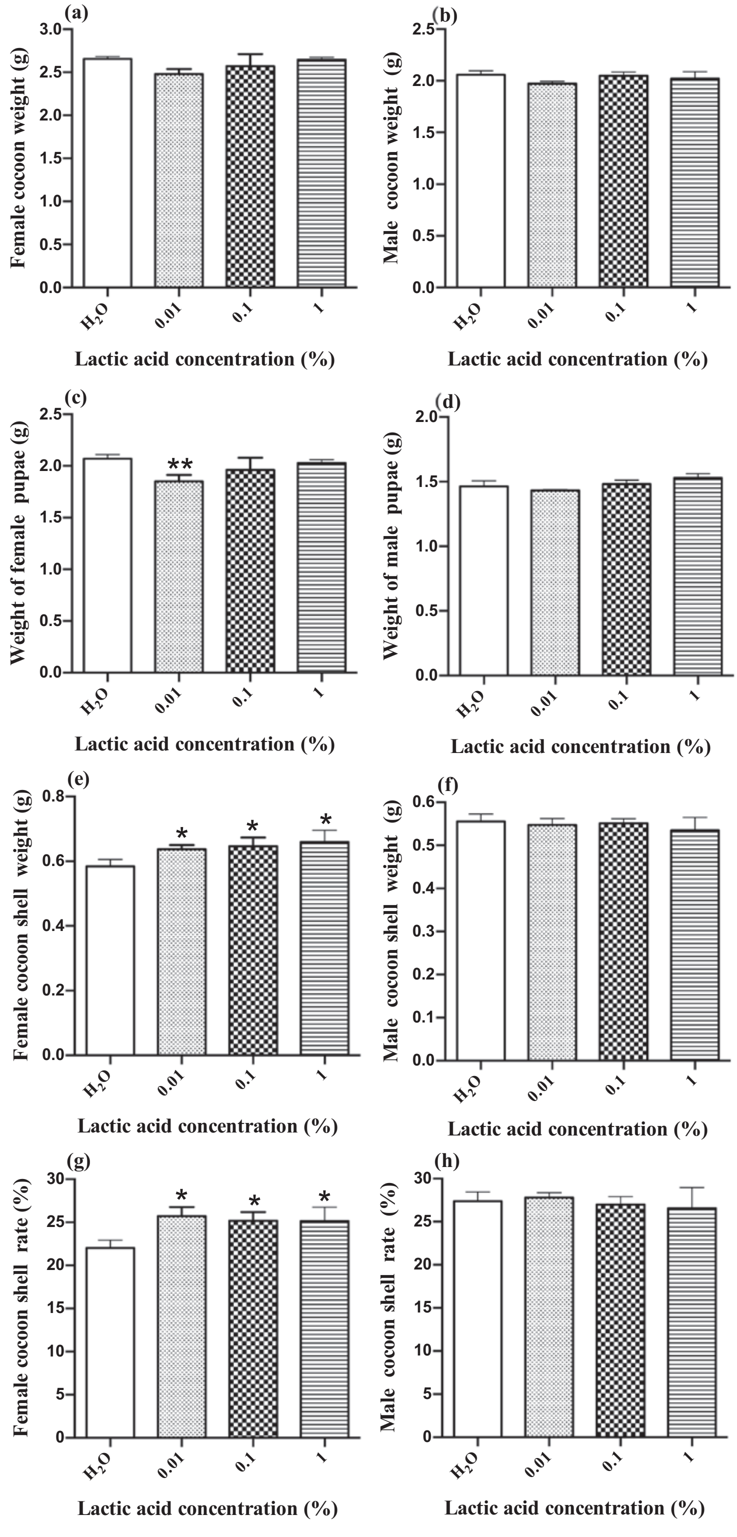


**Fig. 4.** Effects of lactic acid on *Bombyx mori* cocoon production. (a) female cocoon weight; (b) male cocoon weight; (c) female pupal weight; (d) male pupal weight; (e) female cocoon shell weight; (f) male cocoon shell weight; (g) female cocoon shell rate; (h) male cocoon shell rate. Data were presented by means ± SDs ($n$ = 3). *$P$ < 0.05.

supplementation should be readily adopted by sericulture industry for better economic incentive.

In summary, a lactic acid concentration of 0.01–1% increases *B. mori* larval weight, female cocoon weight, and female cocoon shell rate. In contrast, 10% lactic acid negatively affects larval growth, leading to death. Therefore, a low concentration of lactic acid enhances larval growth, while a higher concentration leads to toxicity. The results provide a basic reference for the optimal amount of preservatives. Along with economic growth, sustainable agricultural development and food safety have become the themes

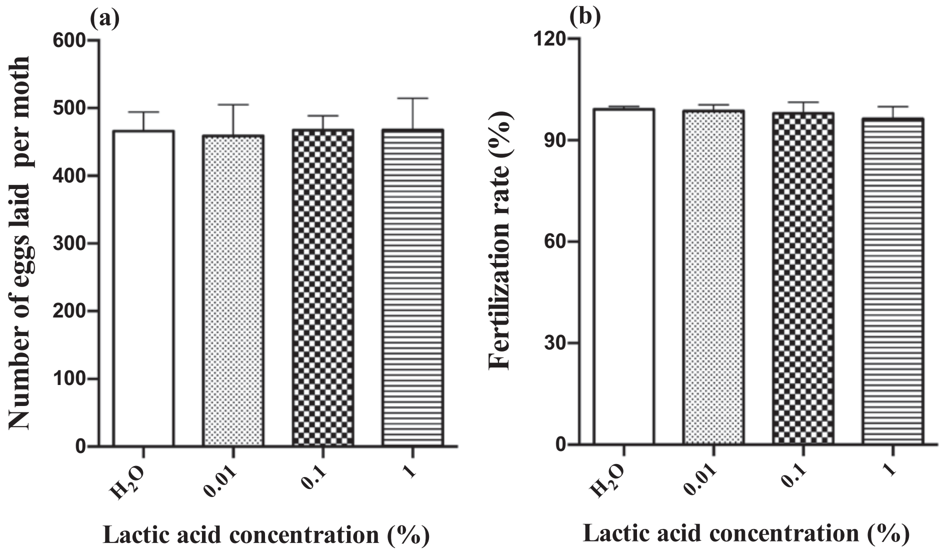


**Fig. 5.** Effects of lactic acid on *Bombyx mori* reproduction. (a) number of eggs laid per moth; (b) fertilization rate. Data were presented by means ± SDs ($n = 4$).

of social development, and food additive safety is a key part of food safety. Additionally, silkworms are sensitive to environmental changes. This study presents a new experimental animal research on environmental quality and food safety and provides reference materials for the dosage safety of food additives.

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## Author Contributions

ZH: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing-original draft. FY: Investigation; Methodology. D-CL: Investigation; Methodology. D-SC: Formal analysis. FW: Conceptualization; Writing-review & editing.

## References Cited

**Ageitos, J. M., A. Pulgar, N. Csaba, and M. Garcia-Fuentes. 2019**. Study of nanostructured fibroin/dextran matrixes for controlled protein release. Eur. Polym. J. 114: 197–205.

**Beales, N. 2004**. Adaptation of microorganisms to cold temperatures, weak acidpreservatives, low pH, and osmotic stress: a review. Compr. Rev. Food Sci. F. 3: 1–20.

**Bhatti, M. F., N. Shahzadi, H. M. Tahir, S. Ali, M. Zahid, and R. Khurshid. 2019**. Effect of honey (*Apisdorsata* [Hymenoptera: Apidae]) on larval growth and silk cocoon yield of *Bombyx mori* (Lepidoptera: Bombycidae). J. Insect Sci. 19: 1–5.

**Cui, W., S. Zhang, Q. Liu, Y. Wang, H. Wang, X. Liu, and Z. Mu. 2016**. An overview on research and commercialization of artificial diet for silkworm (*Bombyx mori*) in China. Sci. Seri. 42: 0003-0015.

**Dang, T. D., A. Vermeulen, P. Ragaert, and F. Devlieghere. 2009**. A peculiar stimulatory effect of acetic and lactic acid on growth and fermentative metabolism of *Zygosaccharomyces bailii*. Food Microbiol. 26: 320–327.

**Elahi, S., and H. Fujikawa. 2019**. Effects of lactic acid and salt on enterotoxin a production and growth of *Staphylococcus aureus*. J. Food Sci. 84: 3233–3240.

**Gurtler, J. B., and T. L. Mai. 2014**. PRESERVATIVES | traditional preservatives - organic acids. Encyclopedia of Food Microbiology (Second Edition) 119–130.

**Huang, X. Z., J. Qin., and Z. H. Xiang. 2013**. The effort to revitalize silk industry in Japan and its inspiration to the transformational development of Chinese silk industry. Sci. Seri. 39: 0599–0605.

**Jiang, L., L. L. Peng, Y. Y. Cao, K. Thakur, F. Hu, S. M. Tang, and Z. J. Wei. 2020a**. Effect of dietary selenium supplementation on growth and reproduction of silkworm *Bombyx mori* L. Biol. Trace Elem. Res. 193: 271–281.

**Jiang, L., L. L. Peng, Y. Y. Cao, K. Thakur, F. Hu, S. M. Tang, and Z. J. Wei. 2020b**. Transcriptome analysis reveals gene expression changes of the fat body of silkworm (*Bombyx mori* L.) in response to selenium treatment. Chemosphere. 245: 125660.

**Khyade, V. B., and S. Yamanaka. 2018**. Sericin from the cocoons of Silkworm, *Antheraea Mylitta* (L) and *Bombyx Mori* (L) for the reduction in hydrogen peroxide induced oxidative stress in feline fibroblasts. Int. J. Sci. Res. Chemi. 3: 1–16.

**Li, Y. J., W. Z. Su, K. K. Hu, P. C. Li, W. Liu, and H. Yao. 2017**. *Lactobacillus plantarum* promotes the growth and development of *Drosophila melanogaster*. Acta Entomol. Sin. 60: 544–552.

**Nishida, S., Y. Ono, and K. Sekimizu. 2016**. Lactic acid bacteria activating innate immunity improve survival in bacterial infection model of silkworm. Drug Discov. Ther. 10: 49–56.

**Saviane, A., L. Toso, C. Righi, C. Pavanello, V. Crivellaro, and S. Cappellozza. 2014**. Rearing of monovotine strains of *Bombyx mori* by alternating artificial diet and mulberry leaf accelerates selection for higher food conversion efficiency and silk productivity. Bull. Insectology. 67: 167–174.

**Shifa, V. J. 2016**. Influence of supplementation of Zea Mays Flour on the growth and economic traits of silkworm, *Bombyx Mori* L. (PFD). IRA-Int. J. Appl. Sci. 3: 469–478.

**Smitha, S., and A. V. B. Rao. 2010**. Effects of selenium on the physiology of heart beat, oxygen comsumption and growth in silkworm *Bombyx mori* L. American-Eurasian J. Toxicol. Sci. 2: 215–219.

**Steib, B. M., M. Geier, and J. Boeckh. 2001**. The effect of lactic acid on odour-related host preference of yellow fever mosquitoes. Chem. Senses 26: 523–528.

**Ude, A. U., R. A. Eshkoor, R. Zulkifili, A. K. Ariffin, A. W. Dzuraidah, and C. H. Azhari. 2014**. *Bombyx mori* silk fibre and its composite: a review of contemporary developments. Mater. Des. 57: 298–305.

**Yang, Q. H., H. Yang, P. P. Li, D. Yang, S. Zhu, and F. Zhu. 2016**. Main types and research status of preservative used in artificial diet of silkworm in China. Anim. Husbandry Feed Sci. 37: 96–97.