Pakistan J. Zool., vol. 51(4), pp 1555-1560, 2019. DOI: http://dx.doi.org/10.17582/journal.pjz/2019.51.4.1555.1560

Effects of Mulberry Leaves with *Bacillus subtilis* on Growth and Production Performance of Silkworm, *Bombyx mori* L.

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Article Information
Received 12 December 2015
Revised 12 May 2018
Accepted 27 August 2018
Available online 28 May 2019

Authors' Contribution
WZ, GP and LC performed the experiments and wrote the manuscript. YM and SL helped in the experimental work. ZH provided technical support.

Key words
***Bacillus subtilis*, Silkworm *Bombyx mori* L., Growth performances, Production performance.**

ABSTRACT

The study was conducted with 1500 healthy silkworm larvae. There were two treatment groups: control group, without added *Bacillus subtilis*, and B.subtilis group, with *B. subtilis* at 2.0×10^{10} CFU/mL added to the feed. Experimental diets were fed in three phases: 3rd instar, 4th instar and 5th instar. Furthermore, 3 replications were maintained and 250 silkworm larvae were reared in each replication. Different growth parameters and cocoon parameters were measured at each stage of silkworm. With the aim to investigate the supplemental effects of B.subtilis on growth performances, cocoon quality and amylase activity of silkworm, Bombyx L, we got the following results: (1) In the larval duration of 3rd instar silkworm, average weight gain (AWG) and feed conversion ratio (RFC) were not significantly affected with the use of *B. subtilis*, but the remaining amount of mulberry leaves (RAML) was significantly decreased contrasting to the blank group ($P<0.05$). (2) In the larval duration of 4th instar silkworm, adding *B. subtilis* to the feed could significantly increase the AWG and RFC and decrease of RAML ($P<0.05$) (3) In the larval duration of 5th instar silkworm, maximum growth parameters level were achieved with silkworm in the experiment group, that the AWG, RFC and RAML, were very significantly affected by adding the *B. subtilis* to the feed ($P<0.01$). (4) Pupa weight (PW) and cocoon weight (CW) were significantly affected by the added *B. subtilis* ($P<0.05$). Other cocoon parameters, such as frison weight (FW) and average cocoon weight (ACW), were more pronounced increased than the blank group ($P<0.01$). (5) The proteinase activity in middle intestine was higher than that of the blank group ($P<0.05$). Collectively, the results indicated that adding B.subtilis to the feed of silkworm had positive effects on growth performances of the Bombyx mori L. apparently.

INTRODUCTION

Silkworm has been reared as a special economic animal more than 5,000 years in China. Sericulture has made a great contribution to the national income of Chinese. However, the low rate of feed conversion had puzzled us in the production of sericulture. Besides that, the phenomenon of severe reduction output of silkworm caused by disease was also the main reason for the low utilization rate of silk. Therefore, we can reduce the occurrence of disease and improve the rate of feed conversion through various methods to overcome this urgent problem. Besides the enhancement of silkworm-breeding techniques and the genetic improvement of variety, it's vital to regulating and improving the intestine-microenvironment of silkworm. It make sense to study the effects of micro-ecological preparation on silkworm.

Earlier studies have shown that the main additives to the feeds are Vitamin C (VC), Potassium iodide, some hormones and other components. It had been found that the content of silk and the rate of feed conversion was enhanced to a certain extent compared with the control group in the studies of silkworm breeding by adding with the VC (Elkaraksy, 1990). Similarly, micro-ecological preparation can also be used as feed additives in silkworm rearing. In the study of foreign scholars whose exploration aims at improving the growth performance of silkworm and enhancing the economic benefit by using various micro-ecological agents can get these aims (Fukumori *et al*., 1989). A large number of microorganisms which making up the intestinal micro-ecology system dwell in

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0030-9923/2019/0004-1555 $ 9.00/0

the gut of insect. The intestinal microflora is beneficial for the host. They can promote the recovery and stabilization of ecological balance of the gastrointestinal tract, maintain the host health and reduce the disease by participating in insect's digestion, absorption and the invasion of pathogenic microorganisms (Dillon *et al*., 2005; Rio *et al*., 2004). Back in the 1960s, Takizawa and Iizuka (1968) had carried out some research on the intestinal bacteria of silkworm. Then there were some scholars found that there are abundant bacteria types in the intestinal of *Bombyx mori* L., in which the predominant bacteria are *Bacillus subtilis* and micrococcus. The *B. subtilis* can secrete protease, amylase, lipase and cellulase, which can help to provide necessary nutrients for the growth of silkworm (Aly *et al*., 2008). As a result, the above researchers offered the possibilities to application of *B. subtilis* as a kind of feed addictive to silkworm rearing.

B. subtilis is a Gram-positive bacterium, which has been widely used as feed additives in livestock. In addition, this organism can increase the animal immunity, reduce intestinal disease and make a complement for the insufficient intestinal enzyme (Schallmey *et al*., 2004). There is a microbial flora in the intestinal of animal, in which the quantity of prebiotics were in a dominant position generally. The dynamics changes of environment can cause the changes of the flora and the imbalance of the micro-ecological. However, the imbalance of this formulation was resumed after the supplementation of the prebiotics agents. On the other hand, the use of micro-ecological agents can increase the number of beneficial microorganisms, which can improve the micro-ecological environment of host.

Considering the advantages of *B. subtilis*, it can be used as a kind of feed additives in sericulture industry. However, there is not much information involved in feeding silkworm with *B. subtilis*. The objective of our study was to investigate the effects of *B. subtilis* added to mulberry leaves on growth and production performances of the silkworm.

MATERIALS AND METHODS

Isolation of digestive protease producing bacteria

Jing Song×Hao Yue hybrid variety of the silkworm, *Bombyx mori* L used in the present investigation were obtained from the College of Life Science, Anhui Agricultural University, Hefei, China. The bacteria were isolated by dissecting the larvae according to the standard isolation method as advocated by Wang *et al*. (2016).

Extraction of intestinal protease from silkworm and activity assay

Each group had a total of 5 frozen silkworms. Each silkworm that had been thawed in deionized water was fixed on the anatomical plate with a pin. The intestines were taken out and the intestinal contents were obtained after the wall was cut with the help of a scissors. A little deionized water was added to the intestine contents and was immediately centrifuged and then supernatant was separated and frozen at -4°C until assayed. The intestine enzyme activity of silkworm was determined by Folin-phenol method using 1% casein as substrate (Lowry *et al*., 1951).

Experimental design and management

A total of 1500 healthy larvae were randomly divided into 2 groups according to similar body weight (with 3 replications). The larvae were subjected to a 2-period feeding program consisting of growing (0 to 2rd instar) and experimental (3rd to 5th instar) periods. One-group served as the control and was fed on mulberry leaves during the entire period. The other group was fed with a similar diet in which the *B. subtilis* preparation was added during the experimental period. In the 3rd to 5th instar, the volume of *B. subtilis* additive chosen in this study were 135 ml (the concentration of bacteria was 2.0×10^{10} CFU/mL) and shown in Tables I and II, respectively. Each group has a total of 5 intestines were dissected and the intestine enzyme activity of silkworm was determined by Folin-phenol method using 1% casein as substrate (Lowry *et al*., 1951).

Growth performance and cocoon quality measurement

Average final weight (AFW), average weight gain (AWG), silkworm excrement quantity (SEQ), remaining amount of mulberry leaves (RAML), feed conversion ratio (FCR) and total residual amount (TRA) were recorded on 3rd to 5th instar. Cocoon parameters like pupal weight (PW),

Table I.- Volume of *Bacillus subtilis* additive and bacterial enzyme activity in the 4th instar.

Program	1d	2d	3d	4d	5d	Total (average) quantity (activity)
Volume of bacteria (ml)	22.50	129.00	123.00	112.50	--	387.00±16.66
Proteinase activity (u/ml)	17.47	12.64	11.84	7.01	--	12.24±1.43

--, means no spraying, and each group of experiment groups has the same quantitative volume of bacteria liquid with the above-mentioned table; ± means SD (standard deviation).

frison weight (FW), shell weight (SW), cocoon weight (CW), average shell weight (ASW), average cocoon weight (ACW), shell ration (SR) were taken into consideration.

Experimental data analysis

Statistical analysis was taken by SPSS 20.0 software, by using Duncan's multiple comparisons, with $P<0.05$ and $P<0.01$ as significant difference and very significant judgment standard. Each value was expressed as the Mean±Standard Error (SE) of each separate observation.

RESULTS

Effect of B. subtilis *on the growth of silkworm*

3rd instar

The effect of feeding *B. subtilis* on average weight level, feed conversion ratio, excrement quantity and remaining amount of mulberry leaves of third instars are presented in Table III. Comparison of different experimental diets showed that the average weight level had elevated 9.73% and the remaining amount of mulberry leaves had decreased by 6.97% than the treatment of blank space, which showed significant difference ($P<0.05$). The addition of *B. subtilis* had no remarkable effect ($P>0.05$) on the average weight gain and silkworm excrement quantity and feed conversion but improved by 7.77%, 10.39% and 6.72%, respectively compared with the blank group ($P>0.05$).

4th instar

As shown in Table III, the average final weight, the average weight gain and the feed conversion ratio increased by 11.09%, 11.58% and 10.59% than the blank group, respectively. The remaining amount of mulberry leaves was 10.25% lower than that of blank group, which showed significant difference ($P<0.05$). Silkworm excrement quantity increased by 7.19% but there were statistically nonsignificant differences ($P>0.05$).

5th instar

As presented in Table III, silkworm of experiment group had elevated 12.52%, 13.01% and 10.57% than blank group in average final weight, average weight gain and feed conversion ratio, and had decreased 23.70% than blank group in the remaining amount of mulberry leaves. The differences were very significant ($P<0.01$). The addition of *B. subtilis* had no significant effect ($P>0.05$) on the silkworm excrement quantity but improved by 12.15 compared with the blank group ($P>0.05$).

Table II.- Volume of *Bacillus subtilis* additive and bacterial enzyme activity in the 5th instar.

Days	Volume of bacteria (ml)	Enzyme activity of bacteria (u/ml)
1d	116.25	0.36
2d	107.25	0.36
3d	127.50	0.36
4d	142.50	0.36
5d	146.25	0.96
6d	168.75	0.53
7d	157.50	0.16
8d	157.50	1.25
9d	112.50	0.83
10d	30.00	0.74
11d	--	--
Total (average) Quantity (activity)	1266.00±32.88	0.59±0.07

--, means no spraying; ± means SD (standard deviation).

Table III.- Impact of the *Bacillus subtilis* on growth performance of 3rd, 4th and 5th instars. The values are in Mean±SEM.

Project/ Group	3rd instar		4th instar		5th instar	
	Control group	Test group	Control group	Test group	Control group	Test group
AIW(g)	21.74±0.20	24.33±0.24	53.78±0.03	59.58±0.88	201.03±2.16	226.10±1.42
AFW(g)	53.78^b±0.03	59.58 ^{a}±0.88	201.03^B±2.16	226.10^A±1.42	767.12^B±1.61	876.89^A±5.56
AWG(g)	32.05±0.63	34.75±1.62	147.25^b±2.19	166.53^a±2.30	566.10^B±0.55	650.79^A±6.98
SEQ(g)	10.70±0.24	11.94±0.53	61.86±2.25	66.65±1.33	600.34±18.21	683.40±23.24
RAML(g)	32.30^b±0.02	30.05^a±0.28	79.40^b±1.15	76.26^a±1.23	380.93^B±2.83	290.65^A±7.37
RFC (%)	0.52±0.04	0.56±0.28	0.72^b±0.01	0.81^a±0.01	0.64±0.001	0.71±0.006

AIW, average initial weight; AFW, average final weight; AWG, average weight gain; SEQ, silkworm excrement quantity; RAML, remaining amount of mulberry leaves; RFC, feed conversion ratio; SE, standard error; Lowercase superscript of data in the same line indicated significant difference ($P<0.05$). Uppercase superscript of data in the same line indicated great significant difference ($P<0.01$). Same letter or no label indicated no significant difference.

Table IV.- Impact of the *Bacillus subtilis* on the bad cocoon. The values are in Mean±SEM.

Bad cocoon program	Control group	Test group
Satiny cocoon	0.50±0.71	0.00±0.00
Dead worm cocoon	5.00±0.00	0.00±0.00
Macular cocoon	1.00±1.41	0.00±0.00
Fly larvae cocoon	2.00±1.41	4.00±0.00
Coarse cocoon	0.00±0.00	0.00±0.00
Twin cocoon	0.00±0.00	1.00±1.41
Total	8.50±0.71	5.00±1.41

Note: ± means SD (standard deviation).

Effect of B. subtilis *on cocoon quality*

Bad silkworm cocoon

From Table IV we can draw a conclusion that, in the mass bad cocoon in the test group had decreased by 41.18% than the control group. No significant difference shown ($P>0.05$).

Table V.- Impact of the *Bacillus subtilis* on silkworm cocoon and intestinal enzymes of silkworm. The values are in Mean±SEM.

Project/Group	Control group	Test group
Silkworm cocoon		
PW (g)	267.90^{b}±7.20	316.39^{a}±2.57
FW (g)	4.30^{B}±0.14	4.85^{A}±0.22
SW (g)	85.53±3.53	97.82±1.34
CW (g)	353.44^{b}±10.73	414.22^{a}±1.53
ASW (g)	0.40±0.004	0.43±0.006
ACW(g)	1.65^{B}±0.000	1.81^{A}±0.005
SR (%)	0.24±0.003	0.24±0.004
Intestinal enzymes of silkworm		
Enzyme activity value (U/ml)	13.15^{A}±0.17	10.40^{B}±0.17

For abbreviations and statistical details, see Table III.

Silkworm cocoon

As it shown in Table V, PW and CW in experimental group were improved by 15.32% and 14.67% than control group, respectively, which showed significant difference ($P<0.05$). FW and ACW was improved by 11.34% and 8.84%-very significant difference were observed ($P<0.01$) among the processing techniques. As regards SW, ASW and SR, they were observed improved by12.56%, 6.98% and 4.12%, respectively but differed non significantly ($P>0.05$).

Effect of B. subtilis *on the intestinal enzymes*

In Table V, the intestinal enzymes of silkworm in test group had increased by 20.91% than control group, and the difference is significant ($P<0.05$).

DISCUSSION

Growth performances of silkworm

Growth performance can be improved with the diet were supplemented probiotics. Present findings was supported by Lorek *et al*. (2001) who observed that the use of probiotics in diet can improve the utilization of carbohydrate and energy. In this experiment, which can be concluded from Table III that adding *B. subtilis* to the mulberry leaves were effective in improving the growth performance of silkworm, the result fall in line with Sanders (1999) and another scholars' research on probiotics can improve the utilization efficiency of feeding living animals. In addition, Teo and Tan (2006) showed that *B. subtilis* can improve the daily gain and the feed conversion ratio of 42 days broilers. Kyriakis *et al*. (1999) in the study of feeding piglet with 10^6 cfu/g or 10^7 cfu/g *B. subtilis* microbial ecological agents shows that the piglet the average daily gain and feed conversion rate was significantly better than the control group.

Cocoon quality

Larval growth of silkworm was mediated by the probiotics which is ultimately represents in the economic traits namely pupal weight, frison and shell weight, cocoon and shell ratio etc., influencing the productivity and quality. What's more, the quality of the cocoon product was largely relyed on the quality of cocoons served to reel, under this circumstance, it's an urgent need for us to improve the quality of cocoon (Guo-Ping and Xi-Jie, 2011). Research has shown that, because of the developed degree of silk gland and the content of silk within the glandular in 5^{th} instar were far more than before this instar larvae, the influence on the growth of silk gland and the synthesis of silk were mostly exerted by the nutrition in 5^{th} instar. The content of organic acid such as amino acid in mulberry leaves has great effect on the quality of silk, it marked that the higher the amino acid content is, the more the rate of feed conversion and the amount of silk will be. *B. subtilis* also has the ability to produce organic acid, synthesis vitamin and resist the fungal in intestine, etc. (Gong *et al*., 2006). That might explain, at least partially, why the amount of silk in test group was significantly higher than that of control group.

Intestinal enzyme activity

Antibiotics seem to trigger some disadvantages as additive in silkworm feed, such as generate drug resistance strains, drug reside, and decrease silkworm's immunity, by largely decreasing the yield and quality of cocoon during metamorphosis (Pusztal *et al*., 1990). Thus, it's an inevitable trend to find nontoxic and non-side-effect natural addictive as substitute of antibiotics for improving the development of modern sericulture (Sonnenschein *et al*., 1993). In the study and application of *B. subtilis* as microbe feed addictive, Cao *et al*. (2009) found exogenous pathogenic bacteria was mostly aerobic strains, *B. subtilis* colonization in intestinal could consume massive oxygen and makes the condition an adverse impact on aerobic bacteria survival, enhancing the number of aerobic-harmful bacteria for reducing. This study illustrated that the experimental group had significant differences ($P<0.05$) in intestinal enzyme activity of silkworm than the control group, suggesting that *B. subtilis* did play a role in improving the stability of intestinal flora of silkworm and promoting intestinal enzyme activity of silkworm .The conclusion was consistent with the previous study, which was conducted by Lin *et al*. (2004). His study displayed that *Bacillus* can improve the digestibility coefficients of nutrients of Litopenaeus vannamei, which could be understood that probiotic can stimulate the intestinal enzyme activity of aquatatic animals and other invertebrates, and the stimulation of enzyme activity was beneficial for the higher digestibility of invertebrates.

Based on the above discussion, it could be inferred that *B. subtilis* was a relatively ideal viable bacteria preparation. This is due to the bacteria strain belongs to the normal flora of silkworm, which can ensure the probiotics quickly enter the intestinal tract for colonization and quickly grow and reproduce; it was partly because of the bacteria strain can tolerate the intestinal environment with low pH and bile. The results of this experiment kept the pace with the effect of using *B. subtilis* as microbial ecological agents applied to poultry, pig and aquatic and it revealed that *B. subtilis* was applied to silkworm feeding as microbial ecological agents, either.

CONCLUSION

Compared with the control group, dietary *B. subtilis* (2.0×1010 CFU/mL) improved the growth performance of silkworm, *Bombyx* L. Therefore, the *B. subtilis* probiotic may be as microbial ecological agents to apply to the feed of silkworm, *Bombyx* L.

ACKNOWLEDGEMENT

This work was jointly supported by the Biology Key Subject Construction of Anhui, the Spark Program of China (2013GA710092). The fund of the State Key Laboratory of Animal Nutrition (2004DA125184F1418, 2004DA125184F1104) and the Fund of National Nature of China.

Statement of conflict of interest

Authors have declared no conflict of interest.

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