Effect of Bacillus Subtilis Protease on Growth, Production Performance and Feed Efficiency of Silkworm, Bombyx Mori L

Linghong Sun
Anhui Agricultural University, Hefei, CN
Anam Khalid
Anhui Agricultural University
Fatima Khalid
Anhui Agricultural University
Zaigui Wang (wangzaigui2013@163.com)
Anhui Agricultural University

Research

Keywords: silkworm, protease, growth performance, feed efficiency, cocoon quality

Posted Date: June 5th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-32668/v1

Abstract

Background: The effects of *Bacillus subtilis* protease on the silkworm (*Bombyxmori L)* production performance and feed efficiency were investigated. 2250 silkworms were randomly divided into two groups, the test group and the control group, each group had three replicates. A certain concentration of protease was sprayed to the mulberry leaves of the test group, while equal volume of deionized water was sprayed to the control group. Test diets were fed in three phases: 3rd instar, 4th instar and 5th instar larval stage.

Results: The results demonstrated that: (i) final weight (FW), average daily gain (ADG), average daily feed intake(ADFI), and feed conversion ratio (FCR) in 4^{th} and 5^{th} larval instar, (ii) midgut protease activity, crude protein digestibility (CPD), crude fiber digestibility (CFD) and cocoon shell conversion rate (CSCR) (iii) the total number of bad cocoons,coarse cocoon, average pupa weight (APW), average cocoon shell weight (ACSW) and average cocoon weight (ACW) were extremely significantly higher ($P<0.01$) in test group as compared to control group. While, (i) the digestibility of crude lipid (EED) and (ii) the fly larvae cocoons in test group were higher significantly ($P<0.05$) than that of the control group.

Conclusion: These results indicated that the *B. subtilis* protease could enhance the silkworm growth performance, feed efficiency and the cocoon quality of silkworm.

1. Introduction

Bombyx mori belongs to the *Lepidoptera silkworm moth*, which is known as one of the important economic insect for producing fine quality silk (1). Sericulture is a long established traditional industry in Asia, and some people's lives are inseparable from this industry. *B.mori* mainly feeds on mulberry leaves, which is fast growing and important revenue crop. Mulberry leaves provides the important nutrients to perform the physiological processes in *B.mori* insect (2)

To enhance the quality and quantity of silkworm cocoon is conducive to improve economic benefits of cocoon industry and hence meet the production needs. Nutritional intake has an important effect on overall physiology such as weight of larva and cocoon, silk production rate and other reproductive traits (3). Because the feed efficiency is the ratio of product to feed used, which is directly related to input and output in production. Therefore, to improve the efficiency of silkworm feed, quality and quantity of cocoon has always been a great concern of the industry. Advancements in silkworm nutrition and synthesis of artificial diet supplemented with the trace elements (1, 2), like, amino acids (4), vitamin (5, 6), and hormone-like substances (7), will be of great benefit to the silk industry (1). It shows that addition of various supplements to silkworm diet can improve the economic traits and silk conversion rate, which includes development of silk gland, digestibility of mulberry leaves and cocoon shell. It was suggested that absence of L-ascorbic acid from silkworm diet, especially in the 1st and the last instars, had a useful impact on the production of cocoon, without effecting the survival rate and larval cycle (8). Many other studies shows that feed additives can prevent disease (9, 10). Various groups of feed supplements, which

includes enzymes, prebiotics, organic acids, essential oils and immune stimulants have been evaluated, to check the potential of antibiotic growth promoter with a variable degree of success(11–13).

Proteases is considered among the most economic and valuable enzyme which cover about 60% of the whole enzyme market (14). Protease can catalyze the hydrolysis of protein and polypeptide, which can be very effective for animal life. Because of ongoing increase in cost of feed ingredients, resulted in increased interest of using enzymes in animal feed. Adding exogenous protease can reduce the detrimental effects of anti-nutritional factors (ANF) on non-ruminants (15), and effectively improve the growth performance, nutrients digestibility, crude protein (CP) and amino acid (AA) (16). Particularly in the gut of infant animals, where the working ability of endogenous protease is not optimal, adding exogenous protease to the diet is better solution (17, 18). The increased nutritional effectiveness of enzyme supplementation is associated with the increased energy, protein and nutrients digestibility. Chicks fed with protease-added corn-soybean meal based diet led to an increase in ADG, but the FCR without any change(19).

Microbial protease is a natural protein digestive enzyme that can decompose proteins and protein-like anti-nutritional substances stored in different parts of the plants (20, 21), thereby increasing bioavailability of the nutrients. The efficiency of exogenous protease as a feed enzyme was undoubtedly testified in recent few years (16). Apparently, every microbial enzymes target different anti-nutritional substances in feed and exhibit cumulative effects by increasing the discharge of nutrients in the diet. The bacilli species provides 70% of the total protease and this ability has made these organisms to be focused by the researchers in the field of biotechnology (22). *Bacillus subtilis* is a potent producer of extracellular degrading enzymes that may be provided to the animal in situ to aid nutrient digestion. Exogenous enzymes, such as carbohydrate enzymes, phytase as well as protease, are widely accepted to enhance the digestibility of nutrients (23, 24). The supplementation of single exogenous protease to the diet can improve the amino acid profile and the protein digestibility of low protein feeds (25, 26). At present, there are many reports on the use of protease as an additive in livestock and poultry feeds with their growth and production performance have been improved (17, 20), but there are few studies on the use of *B. subtilis* protease as the feed additive of silkworm.

Therefore the present study reports the effect of mulberry leaves supplemented with the *B. subtilus* extracted protease enzyme. The main focus was to examine the effect on growth, production performance and feed efficiency of silkworm.

2. Materials And Methods

2.1. Silkworms and experimental treatments

Protease producing strain used in the experiment was *B. subtilis* isolated from the intestinal tract of silkworm. The mulberry leaves were collected from the experimental teaching base of Anhui Agricultural

University. The test silkworm species (Jingsong × Haoyue) were provided by Silkworm Laboratory of Anhui Agricultural University, and were conventionally-raised to the end of the second instar larval phase.

2.2. Preparation of crude enzymes

Bacterial species was allowed to grow for 36 h hours and then broth was centrifuged at 5000 rpm, for 10 minutes, at 4 °C. After that solid ammonium sulfate $(NH_4)_2SO_4$ was added slowly to the filtrate to get 80% saturation. The filtrate was allowed to stand for 12 hours at 4 °C, until the protease was separated. Refrigerated centrifugation was done to get the precipitates, following the dilution of precipitates by adding small amount of deionized water (Ratio of crude enzyme to pure water was approximately 1:5), and finally the crude enzymes were prepared.

2.3. Silkworms feeding protocol

A total of 2250 healthy and similar weight 2nd instar silkworms (Jingsong × Haoyue) were selected and randomly divided into two groups: *B. subtilis* protease group (test group) and non-added *B. subtilis* protease group (control group). Each group had three replicates, with 375 silkworms in each repeat.

Rearing bed was disinfected with lime powder $Ca(OH)_2$. Fed 3 times a day: 8:00, 14:30 and 20:30. Routinely reared to 3rd instar, weighed and grouped. Fresh mulberry leaves were weighed, recorded and fed to the silkworm. The feeding experiment was started from the start of 3rd instar to the end of the 5th instar. Silkworms were weighed at the beginning and end of the 3rd, 4th and 5th instar. There were three days in the 3rd instar, five days in the 4th instar, and eleven days in the 5th instar. The protease liquid was evenly sprayed on the surface of fresh mulberry leaves for the test group, similarly equal volume of distilled water was evenly sprayed on fresh mulberry leaves for control group. Silkworms were allowed to feed after the mulberry leaves properly dried at room temperature. The total amount of enzyme activity of *B. subtilis* enzyme solution that was sprayed daily on the mulberry leaves of the test group, is shown in Table1. Residual leaves and feces were removed regularly, to avoid contamination.

Table 1
Test group enzyme solution spraying amount and enzyme activity.

Days	Enzyme volume (ml)	Enzyme activity (U/ml)
3rd instar		
1d	15.0	4.802
2d	15.0	4.802
3d	—	—
4th instar		
1d	15.0	6.692
2d	86.0	4.827
3d	82.0	4.634
4d	75.0	5.958
5th instar		
1d	77.5	2.066
2d	71.5	1.280
3d	85.0	1.742
4d	95.0	1.571
5d	97.5	3.122
6d	112.5	1.310
7d	105.0	0.518
8d	105.0	2.990
9d	75.0	2.831
10d	20.0	2.719
11d	—	—

2.4. Sample collection and analytical determination

1. Determination of moisture content of mulberry leaves: 30 g of fresh mulberry leaves were randomly taken, dried at 95°C, and the moisture content of mulberry leaves was calculated.
   Enzyme activity assay: Using Folin-phenol reagent method and casein as substrate, the content of amino acid production was determined by spectrophotometer, thereby determining the enzyme activity of *B. subtilis* protease solution (27).

Growth performance and feed efficiency: All silkworm excrement and remaining mulberry leaves from each repeat were collected at the end of 3rd, 4th and 5th instar respectively, weighed and recorded to calculate the conversion rate of mulberry leaves and feed digestibility. At the end of 5th instar, cocoons were harvested and weighed after 7 days of mature silkworm mounting. Fresh mulberry leaves and silkworm excrement were dried to measure feed conversion rate. According to the method of Association of Official Analytical Chemistry(AOAC) (28), the content of crude protein, crude fiber and crude fat in 5th instar silkworm excrement fed mulberry leaves were determined. The silkworm digestibility (D) of crude protein, crude fiber and crude fat in mulberry leaves were calculated according to the following formula. Final weight (FW), average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR), digestibility of crude protein (CPD), digestibility of crude fiber (CFD), digestibility of crude lipid (EED), cocoon shell conversion rate (CSCR).

$D = (C1 - C2)/C1 \times 100\%$

C1: Nutrient content in mulberry leaves

C2: Nutrient content in excrement of silkworm

1. Cocoon quality: At the end of 5th instar, the silkworm cocoons were harvested after 7 days of mature silkworm mounting, the pupa body, cocoon, frison and cocoon shell were weighed. The number of defective cocoons (including, satiny cocoon mites, dead worm cocoon, macular cocoon, fly larvae cocoon, coarse cocoon and double cocoon) and the total number of cocoon (CN) were counted. The average pupal weight (APW), average frison weight (AFW), average cocoon shell weight (ACSW), the average cocoon weight (ACW) and cocoon shell ratio (CSR) were calculated.

$CSR = CSW/CW*100\%$

$ACW = APW + ACSW$

1. Silk glands weight and intestinal protease activity: On the 8th day of the 5th instar, 4 silkworms were randomly selected from each replicate, weighed and temporarily refrigerated at -20℃ for 18 hours to be dissected. Silk glands and midgut of both (test group and control group) were taken out after dissection and, and the weight of silk glands was measured. The midgut was mashed in a small beaker, and a little amount of pH 7.0 buffer (Na_2HPO_4-NaH_2PO_4) was added, then centrifuged at 5000 rpm, for 10 minutes at 4 °C. Then supernatant was taken, volume was adjusted to 100 mL with a buffer of pH 7.0, and the protease activity of the middle intestinal juice was determined by the Flin-phenol reagent method (Lowry et al., 1951).

2.5. Data analysis

All data analysis was first done by Excel and then subjected to statistical analyses which were performed by using one-way ANOVA procedures of SPSS 18.0. Differences between test group and control group were considered significant at $p < 0.05$ and extremely significant at $p < 0.01$, and were expressed as mean ± SD.

3. Results

3.1. Effect of B. subtilis protease on the growth performance of silkworm

Fresh mulberry leaves were dried and the moisture content of mulberry leaves was measured to be 72.25%. As shown in Table 2, at the 3rd instar, there was no significant difference in FW, ADG, ADFI and FCR between the test group and the control group ($P > 0.05$).

At the 4th and 5th instars, FW, ADG, ADFI and FCR of the test group were higher than that of the control group ($P < 0.01$), which indicates that addition of *B. subtilis* protease can improve all the measured growth parameters. FW, ADG, ADFI and FCR of the test group 4th instar increased by 11.13%, 13.00% and 1.18%, respectively, compared with the control group. While FW, ADG, ADFI and FCR of the test group 5th instar increased 14.36%, 15.52%, 3.65% and 14.44% respectively, compared to the control group. The growth rates of FW, ADG, ADFI and FCR in the 5th instar are all higher than those in the 4th instar.

Table 2
Effect of feeding *B. subtilis* protease on growth performance of 3rd, 4th and 5th instar larva.

Group	FW(g)	ADG(g)	ADFI(g)	FCR (%)
3rd instar				
Control group	56.18 ± 1.75	10.92 ± 0.36	38.97 ± 0.16	30.89 ± 1.37
Test group	59.69 ± 0.91	11.89 ± 0.39	39.58 ± 0.31	33.18 ± 1.23
P	0.065	0.060	0.067	0.154
4th instar				
Control group	203.34 ± 3.07	36.79 ± 0.33	128.07 ± 0.12	32.62 ± 0.39
Test group	225.98 ± 1.00	41.57 ± 0.05	129.58 ± 0.31	36.76 ± 0.21
P	< 0.001	< 0.001	0.003	< 0.001
5th instar				
Control group	770.43 ± 4.50	51.55 ± 0.40	305.42 ± 1.66	20.43 ± 0.32
Test group	881.09 ± 2.61	59.56 ± 0.17	316.56 ± 0.59	23.38 ± 0.25
P	< 0.001	< 0.001	< 0.001	< 0.001

FW, final weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio. n = 3.

3.2. Effect of B. subtilis protease on feed efficiency

As shown in Table 3, CPD, CFD, EED and CSCR in the test group increased by 6.40%, 28.64%, 7.01% and 34.34%, respectively. These values of CPD, CFD and CSCR showed significant difference as compared to the control group ($P < 0.01$). While EED in the test group was higher than that in the control group ($P < 0.05$).

Table 3
Effect of adding *B. subtilis* protease on feed efficiency of silkworm.

Group	CPD(%)	CFD(%)	EED (%)	CSCR(%)
Control group	61.12 ± 0.17	8.38 ± 0.26	58.63 ± 1.20	7.63 ± 0.51
Test group	65.03 ± 0.23	10.78 ± 0.42	62.74 ± 0.49	10.25 ± 0.12
P	< 0.001	0.002	0.011	0.002

CPD, digestibility of crude protein; CFD, digestibility of crude fiber; EED, digestibility of crude lipid; CSCR, cocoon shell conversion rate. n = 3.

3.3. Effect of B. subtilis protease on the quality of silkworm cocoon

As shown in Table 4, compared with the control group, *B. subtilis* protease can increase the number of defective cocoons ($P < 0.01$), among which, the number of coarse cocoons was larger than that in the control group ($P < 0.01$). While test group had a larger number of fly larvae cocoons than the control group ($P < 0.05$). The addition of *B. subtilis* protease had no significant effect on dead worm cocoon, macular cocoon, cotton cocoon and double cocoon ($P > 0.05$).

Table 4
Effect of adding *B. subtilis* protease on bad cocoon.

Bad cocoon program	Control group	Test group	P
Dead worm cocoon	5.00 ± 0.82	3.00 ± 0.82	0.070
Coarse cocoon	0.00 ± 0.00	3.00 ± 0.82	0.007
Macular cocoon	1.00 ± 0.00	2.00 ± 0.47	0.158
Satiny cocoon	0.67 ± 0.47	0.00 ± 0.00	0.116
Double cocoon	0.00 ± 0.00	0.00 ± 0.00	-
Fly larvae cocoon	2.00 ± 0.82	4.67 ± 0.47	0.016
Total	8.67 ± 0.47	12.67 ± 0.47	0.001
n = 3			

The effect of adding *B. subtilis* protease on silkworm cocoon quality was shown in Table 5. The CN in the test group was larger than that in the control group ($P = 0.025$), and the APW ($P < 0.001$), ACSW ($P = 0.009$) and ACW ($P < 0.001$) were higher than those in the control group ($P < 0.01$). The addition of *B. subtilis* protease had no significant effect on the AFW and CSR ($P > 0.05$).

Table 5
Effect of adding *B. subtilis* protease on cocoon quality.

Group	APW (g)	AFW (mg)	ACSW (mg)	ACW(g)	CN	CSR (%)
Control group	0.992 ± 0.027	21.00 ± 1.09	325.7 ± 25.7	1.316 ± 0.026	217.7 ± 3.9	24.78 ± 2.35
Test group	1.345 ± 0.018	21.75 ± 0.13	417.8 ± 10.0	1.763 ± 0.026	229.0 ± 2.4	23.70 ± 0.34
P	< 0.001	0.391	0.009	< 0.001	0.025	0.553

APW, average pupal weight; AFW, average frison weight; ACSW, average cocoon shell weight; ACW, average cocoon weight; CN, cocoon number; CSR, cocoon shell ratio. n = 3.

3.4. Effect of B. subtilus protease on the development of silk gland in silkworm

The weight of silk glands in each group is shown in Table 6. However, the effect of *B. subtilis* protease on the development of silk glands was not significant ($P > 0.05$).

Table 6
Effect of adding *B. subtilis* protease on the weight of silkworm silk gland.

Group	Silk gland weight (g)
Control group	1.012 ± 0.056
Test group	1.029 ± 0.030
P	0.435
n = 10	

3.5. Effect of B. subtilis protease on protease activity in the middle intestine of silkworm

The activity of midgut protease in silkworm was shown in Table 7. The activity of midgut protease in the test group was higher than control group ($P < 0.001$). These results indicate that the addition of *B. subtilis* protease can significantly improve the activity of midgut protease in silkworm.

Table 7
Effect of *B. subtilis* protease on the activity of protease in midgut of silkworm.

Group	Enzyme activity (U/ml)
Control group	12.73 ± 0.55
Test group	15.08 ± 0.92
P	< 0.001
n = 6	

4. Discussion

Levels of protein and the limiting amino acids in the diet severely affects the larval growth. The results have shown that application of *B. subtilis* protease had no significant effects on FW, ADG, ADFI and FCR of the 3rd instar silkworm. It may be because the silkworms were weighed and grouped in the beginning of the experiment, which caused some stress on the silkworm and affected the function of the protease. Another cause may be the period of 3rd instar larvae was too short to show the outcomes of feeding, so the growth performance of the silkworm in the test group was not significantly different from that of the control group. At the 4th and 5th instars, FW, ADG, ADFI and FCR of the test group were extremely significantly higher than those of the control group, indicating that the addition of *B. subtilis* protease could significantly improve FW, ADG, ADFI and FCR of the 4th and 5th instar silkworms. Nutritional tests were mostly carried out by rearing larvae on artificial diets, and the effects of supplementation of specific substance on larval growth, development, and survival were carefully examined(29). Beneficial effect of protease was studied for different animals. A study proposed that exogenous protease could increase the

ADFI and ADG of broilers, but the FCR was not affected (19), similarly it was reported that the supplementation of exogenous protease resulted a decrease in FCR and an increase in weight gain (30) It was also observed that the addition of protease in the growing-finishing period led to a decrease in ADG of pigs (31). Reasons for the difference in results were most likely because of different compositions of diets, dose concentrations and the sources of protease.

It was reported that adding protease in a low protein or high protein diet could improve feed efficiency, they investigated that broiler feed supplemented with a protease could improve the CPD and EED (32). Similarly, our present study found that *B. subtilis* protease could significantly improve CPD, FCR and CSCR. In our test, an increase in the apparent digestibility of crude protein and crude lipid may be one of the reasons for the improvement in growth and production performance. The addition of exogenous protease could increase the hydrolysis of dietary protein, thereby making more peptides and amino acids available for utilization in the small intestine (33, 34). The digestion and absorption of crude protein in feed directly affects the synthesis of silk protein. Therefore, increasing CPD will increase the ability of silkworm to synthesize silk, which can improvement the feed efficiency. This study showed that protease can significantly increase the EED and CSCR of the test group as compared to the control group. However, the results of this study showed that silkworm CFD of the test group was significantly higher than that of the control group. It was reported that probiotics can enhance the enzymatic activity which in turn can enhance the nutritional parameters of silkworm (35).

The results of this experiment showed that the number of dead worm cocoon in the control group was higher than that in the test group. The reason may be that the protease increased the CPD and increased the absorbable amino acids in the mulberry leaves. It was found that amino acids could reduce the total larval duration and mortality of larvae and pupae(36). It was found that during metamorphosis in silkworm, changes in protease activity in the midgut were observed. Especially remarkable increase in the enzyme activity was seen before emergence (37). Since enzymes serves as a reaction catalyst of any biological system. So, the activities of digestive enzymes in silkworm like, alkaline protease and alkaline phosphatase can help in silkworm breeding program for improvement of cocoon characters (38). The addition of *B. subtilis* protease in this experiment increased the total number of defective cocoons. Since the test group was added with *B. subtilis* protease, the survival rate of the silkworm in the experimental group was improved, so the density of the test group was higher than that of the control group, and more silkworm excrement was produced than the control group. The poor environment was the main cause of defective cocoons.

The ability of silkworm to synthesize silk protein directly affects the yield of silk. The quality of silkworm cocoon is evaluated by measuring the CN, CSW and CSR. Under the same PW conditions, the heavier the cocoon shell, the higher the CSR. The CSW is an index of cocoon silk quantity. In *B. mori*, particularly, up to 65% of digested nitrogen is utilized for silk production during the last instar and the level of dietary protein and limiting amino acids in the diet strongly affects larval growth and silk production. In this study, the results of cocoon quality showed that *B. subtilis* protease could significantly increase the APW, ACSW, CN and ACW. This may be related to the increased CPD by *B. subtilis* protease. In the test group,

only the CSR was slightly lower than that of the control group. Because some of the defective cocoon cannot be weighed in the test group, not to be included in the calculation, so the CSR of the control group was slightly higher, but the data of ACSW and the ACW could also indicate that the addition of *B. subtilis* protease could improve the silkworm cocoon quality. It could increase the yield of silk, which is very important for the silk industry.

The results showed that the silkworm had a relatively high degree of digestion and utilization of crude protein and crude lipid components in mulberry leaves. The results were related to the crude protein providing the development and growth of the individual and the accumulation of protein to provide the raw material for the subsequent cocoon. Since the main component of silk is protein, silkworm absorbs the crude protein in mulberry leaves, and some of it is deposited into the silk gland through biochemical reaction in the body, which was used for later cocooning. Insect adipocytes can store a great amount of lipid reserves as cytoplasmic lipid droplets and lipid metabolism is essential for growth and reproduction and provides energy needed during extended non-feeding periods (39). Silkworm spinning is a process of converting amino acids in feed into silk glands and excreting them. The amino acid content in feed and the ability to synthesize silk fibroin will affect the amount of silk. Development of silk gland mainly occur in the 5th instar stage, at this time, the nutrients in the feed directly affect the synthesis of silk. Therefore, the appropriate addition of some physiologically active substances in the 5th instar stage will increase the silkworm's ability to synthesize silk, which is beneficial to improve the feed efficiency. However, the results of this experiment showed that the addition of *B. subtilis* protease had no significant effect on the development of silk gland in silkworm.

The digestive enzymes of the midgut control the digestion and absorption of various nutrients in the feed. Results showed that the addition of *B. subtilis* protease could increase the protease activity of the midgut in the silkworm. It may be because the added protease can induce the secretion of enzymes in the midgut, or that the added protease remains in the intestine. One study reported that the activity of natural protease changes during developmental stages of the silkworm, it decreases after the mature larva period, reaches a peak before emergence, and decreases markedly thereafter. But there was no great change in the protein concentration in the midgut except for the adult stage (37). However results of our study showed that feeding *B. subtilis* protease caused an increase in the protease activity which indicated that the silkworm had higher efficiency in protein absorption. After 3 days of silkworm at 5th instar, the amino acids in the body need to be transported to the silk gland to synthesize silk protein (40, 41). Therefore, after feeding *B. subtilis* protease, the silkworm increased the CPD and CSCR.

In conclusion *B. subtilis* protease can improve the growth performance and FCR of silkworm, and improve its economic benefits. Therefore, *B. subtilis* protease can be used as a feed additive for the sericulture industry.

Declarations

Ethics approval and consent to participate

Compliance with Ethical Standards

Consent for publication

Not Applicable

Availability of data and material

Data is available on demand.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by the Major Science and Technology Program of Anhui Province (17030701035), the Fund of College Natural Science from Anhui Province (KJ2018A0141), Anhui Industry and Technology System of Poultry Science (AHCYTX-10), and the Fund of State Key Laboratory of Animal Nutrition (2004DA125184F1725).

Authors' contributions

Z.W. designed and performed the experiments, as well as helped draft the manuscript. L.S., A.K., and F.K., carried out the laboratory work, participated in data analysis, participated in the design of the study and drafted the manuscript. L.S and A.K collected field data. All authors gave final approval for publication.

Acknowledgements:

Authors acknowledge the financial supporters for this study.

Highlights

- Silkworm production for economic benefits.
- Mulberry leaves were supplemented with protease.
- *subtilus* protease improved the growth of silkworm.

References

1. Akhter BN. Effect of Salt, Nickel Chloride Supplementation on the Growth of Silkworm, Bombyx mori L.(Lepidoptera: Bombycidae) Md. Rezuanul Islam," Md. Abdul Ohayed Ali,"Dipak Kumar Paul," Shaheen Sultana. Journal of Biological Sciences. 2004;4(2):170–2.
2. Jiang L, Peng L-L, Cao Y-Y, Thakur K, Hu F, Tang S-M, et al. Effect of Dietary Selenium Supplementation on Growth and Reproduction of Silkworm Bombyx mori L. Biological trace element research. 2019:1–11.
3. Ramesha C, Lakshmi H, Kumari SS, Anuradha CM, Kumar CS, Cohen A. Nutrigenetic screening strains of the mulberry silkworm, Bombyx mori, for nutritional efficiency. Journal of insect science. 2012;12(1).
4. Laz R. Effects of methionine and tryptophan on some quantitative traits of silkworm, Bombyx mori L. (Lepidoptera: Bombycidae). University Journal of Zoology Rajshahi University. 2009;28:15–9.
5. Das S, Medda A. Effect of cyanocobalamin on protein and nucleic acid contents of ovary of silkworm, Bombyx mori L., during larval, pupal and adult stages of development. Int J Trop Insect Sci. 1988;9(5):641–6.
6. Etebari K, Matindoost L. Application of multi-vitamins as supplementary nutrients on biological and economical characteristics of silkworm Bombyx mori L. Journal of Asia-Pacific Entomology. 2005;8(1):107–12.
7. Kamimura M, Tomita S, Kiuchi M, Fujiwara H. Tissue-Specific and Stage-Specific Expression of Two Silkworm Ecdysone Receptor Isoforms: Ecdysteroid-Dependent Transcription in Cultured Anterior Silk Glands. European journal of biochemistry. 1997;248(3):786–93.
8. Cappellozza L, Cappellozza S, Saviane A, Sbrenna G. Artificial diet rearing system for the silkworm Bombyx mori (Lepidoptera: Bombycidae): effect of vitamin C deprivation on larval growth and cocoon production. Appl Entomol Zool. 2005;40(3):405–12.
9. Jacela JY, DeRouchey JM, Tokach MD, Goodband RD, Nelssen JL, Renter DG, et al. Feed additives for swine: Fact sheets–prebiotics and probiotics, and phytogenics. Kansas Agricultural Experiment Station Research Reports. 2010(10):132–6.
10. Kim K, Lee G, Jang J, Kim J, Kim Y. Evaluation of anti-SE bacteriophage as feed additives to prevent Salmonella enteritidis (SE) in broiler. Asian-Australasian journal of animal sciences. 2013;26(3):386.
11. Griggs J, Jacob JP. Alternatives to antibiotics for organic poultry production. Journal of Applied Poultry Research. 2005;14(4):750–6.
12. Applegate T, Klose V, Steiner T, Ganner A, Schatzmayr G. Probiotics and phytogenics for poultry: Myth or reality? Journal of Applied Poultry Research. 2010;19(2):194–210.
13. Caly DL, D'Inca R, Auclair E, Drider D. Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: a microbiologist's perspective. Frontiers in microbiology. 2015;6:1336.
14. Fufeungsombut E, Chim-anage P, Promboon A, Suwannaphan S, editors. Isolation and selection of silk degumming protease producing bacteria from Thailand. 47 Kasetsart University Annual Conference, Bangkok (Thailand), 17–20 Mar 2009; 2009.

15. Munir K, Maqsood S. A review on role of exogenous enzyme supplementation in poultry production. Emirates Journal of Food and Agriculture. 2013:66–80.
16. Cowieson AJ, Roos FF. Toward optimal value creation through the application of exogenous mono-component protease in the diets of non-ruminants. Anim Feed Sci Technol. 2016;221:331–40.
17. Lewis Ca, Catron D, Liu C, Speer V, Ashton G. Swine nutrition, enzyme supplementation of baby pig diets. J Agric Food Chem. 1955;3(12):1047–50.
18. Mahagna M, Nir I, Larbier M, Nitsan Z. Effect of age and exogenous amylase and protease on development of the digestive tract, pancreatic enzyme activities and digestibility of nutrients in young meat-type chicks. Reproduction Nutrition Development. 1995;35(2):201–12.
19. Yuan L, Wang S, Wang Z, Zhu H, Huang K. Effects of exogenous protease supplementation on endogenous trypsin activity and gene expression in broilers. Genet Mol Res. 2015;14(4):13633–41.
20. Barletta A. Introduction: current market and expected developments. Enzymes in farm animal nutrition. 2011:1–11.
21. Ao T. Using exogenous enzymes to increase the nutritional value of soybean meal in poultry diet. Soybean and nutrition InTech, Rijeka, Croatia. 2011:201 – 14.
22. Rahman RNZA, Razak CN, Ampon K, Basri M, Zin WM, Yunus W, et al. Purification and characterization of a heat-stable alkaline protease from Bacillus stearothermophilus F1. Appl Microbiol Biotechnol. 1994;40(6):822–7.
23. Cowieson A, Ravindran V. Effect of exogenous enzymes in maize-based diets varying in nutrient density for young broilers: growth performance and digestibility of energy, minerals and amino acids. British poultry science. 2008;49(1):37–44.
24. Mahmood T, Mirza M, Nawaz H, Shahid M, Athar M, Hussain M. Effect of supplementing exogenous protease in low protein poultry by-product meal based diets on growth performance and nutrient digestibility in broilers. Anim Feed Sci Technol. 2017;228:23–31.
25. Angel C, Saylor W, Vieira S, Ward N. Effects of a monocomponent protease on performance and protein utilization in 7-to 22-day-old broiler chickens. Poultry science. 2011;90(10):2281–6.
26. Law F, Zulkifli I, Soleimani A, Hossain M, Liang J. Nutrient digestibility of broiler chickens fed on a low-protein diet supplemented with mono-component proteases. Eur Poult Sci. 2015;79(79):107.
27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of biological chemistry. 1951;193:265–75.
28. Lee M. Official methods of analysis of AOAC International (16th edn): edited by Patricia A. Cunniff, AOAC International, 1995. $359.00 (North America)/$399.00 (elsewhere)(xxvi + 1899 pages) ISBN 0 935 584 54 4. Elsevier; 1995.
29. ITO T. Nutritional requirements of the silkworm, Bombyx mori L. Proceedings of the Japan Academy. 1967;43(1):57–61.
30. Cowieson A, Toghyani M, Kheravii S, Wu S, Romero L, Choct M. A mono-component microbial protease improves performance, net energy, and digestibility of amino acids and starch, and

upregulates jejunal expression of genes responsible for peptide transport in broilers fed corn/wheat-based diets supplemented with xylanase and phytase. Poultry science. 2018;98(3):1321–32.

31. O'Shea C, Mc Alpine P, Solan P, Curran T, Varley P, Walsh A, et al. The effect of protease and xylanase enzymes on growth performance, nutrient digestibility, and manure odour in grower–finisher pigs. Anim Feed Sci Technol. 2014;189:88–97.
32. Freitas D, Vieira S, Angel C, Favero A, Maiorka A. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono-component protease. Journal of Applied Poultry Research. 2011;20(3):322–34.
33. Guggenbuhl P, Waché Y, Wilson J. Effects of dietary supplementation with a protease on the apparent ileal digestibility of the weaned piglet. Journal of animal science. 2012;90(suppl_4):152–4.
34. Yu G, Chen D, Yu B, He J, Zheng P, Mao X, et al. Coated protease increases ileal digestibility of protein and amino acids in weaned piglets. Anim Feed Sci Technol. 2016;214:142–7.
35. Esaivani C, Vasanthi K, Bharathi R, Chairman K. Impact of probiotic Saccharomyces cerevisiae on the enzymatic profile and the economic parameters of Silkworm Bombyxmori L. Adv Biol BioMed. 2014;1(1):1–7.
36. Krishnappa J. Influence of Amino Acids Supplementation on Growth and Development of Mulberry Silkworm (Bombyx Mori L.): University of Agricultural Sciences, GKVK; 1987.
37. Eguchi M, Furukawa S, Iwamoto A. Proteolytic enzyme in the midgut of the pharate adult of the silkworm, Bombyx mori. J Insect Physiol. 1972;18(12):2457–67.
38. Lakshmikumari B. Effects of gamma radiation on the cytological, physiological and biochemical aspects of silkworm Bombyx mori L: Ph. D. Thesis, Bangalore University, Bangalore, India; 1995.
39. Arrese EL, Soulages JL. Insect fat body: energy, metabolism, and regulation. Ann Rev Entomol. 2010;55:207–25.
40. Tojo S, Kiguchi K, Kimura S. Hormonal control of storage protein synthesis and uptake by the fat body in the silkworm, Bombyx mori. J Insect Physiol. 1981;27(7):491–7.
41. Chen X, Shao Z, Knight DP, Vollrath F. Conformation transition kinetics of Bombyx mori silk protein. Proteins: Struct Funct Bioinf. 2007;68(1):223–31.