EGYPTIAN ACADEMIC JOURNAL OF

BIOLOGICAL SCIENCES

ENTOMOLOGY

A

Vol. 12 No.2 (2020)

ISSN
1687-8809

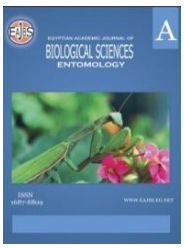
WWW.EAJBS.EG.NET

Vol. 18 No. 1 (2025)

Egypt. Acad. J. Biolog. Sci., 18(1):29-38 (2025)

Egyptian Academic Journal of Biological Sciences
A. Entomology

ISSN 1687- 8809
http://eajbsa.journals.ekb.eg/



Chlorella vulgaris, Spirulina platensis and *Azolla caroliniana* as Sustainable Protein Sources For Feeding Silkworm (*Bombyx mori* L.)

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ARTICLE INFO

Article History
Received:17/12/2024
Accepted:22/1/2025
Available:26/1/2025

Keywords:
Silkworm, probiotics, Chlorella, Azolla, Cryptogams.

ABSTRACT

Good nutrient additives for silkworms constitute a key part of the good quality and quantity of silk produced. By increasing silk output, sericulture can become more economically sustainable. This study delved into the potential of unconventional and sustainable protein sources such as Chlorella, Spirulina and Azolla as supplementary feed for mulberry silkworm. Our findings indicate that all additives positively influenced increased the efficiency of larvae and moth growth and productivity, improved the production of silk and other biological and economic characteristics of *Bombyx mori* L.. However, Chlorella proved to be the most effective, significantly boosting silk gland weight, shell weigh, raw silk and the amount of fibroin in the cocoons, which are very important economic factors in silk production. Supplementing mulberry leaves with sustainable protein sources such as algae or fern is the most promising techniques to increase the economic value of cocoons and improve the production of silk without much investment.

INTRODUCTION

Sericulture is a sustainable initiative that contributes to the social, economic, and environmental aspects of sustainable development. Through mulberry cultivation and silkworm farming, often in rural communities, it provides a means of subsistence, then the silk filament manufactured into various textiles and clothing items. To achieve higher yields of superior quality silk and revitalize the silk industry, it is imperative to provide silkworms with optimal nutrition. This can be achieved through meticulous care of mulberry trees or by supplementing mulberry leaves with nutritional additives. Supplements have the potential to significantly improve larval growth, cocoon production, and consequently, the quality and quantity of the raw silk. In recent years, fortifying mulberry leaves with supplements and assessing their impact on metabolic activities, physiological processes, and silk production has become a promising approach that captivates researchers in the field of sericulture. (Ismail, *et al.*, 2016; Borah and Boro, (2020); He *et al.*, 2021; Mohanny *et al*., 2022; Udayan and Kumar 2022 and Shah *et al.*, 2024). Algae are good sources of protein, fats, carbohydrates, phycobiliproteins, polysaccharides, fiber, chlorophyll, carotenoids, sterols, vitamins, and minerals. Raposo *et al*. (2013). Moreover, it can be used as a supplement for the nutrition of silkworm. Kumar *et al*. (2019). Spirulina (*Spirulina platensis*) is a blue–green micro algae, which rich in protein, vitamins (especially B vitamins), minerals, and essential amino acids. Anvar and Nowruzi (2021). It contains 18 amino acids and vital vitamins such as tocopherol, biotin, thiamine, riboflavin, folic acid, niacin, pyrodozoic acid,

beta-carotene and vitamin B12. It has been associated with boosting immunity, improving digestion, and aiding weight management. Spirulina contains an adequate degree of protein, fat, and micronutrients to increase insect sustenance through direct utilization and indirect use in silkworm nourishment. Hassan (2020) and Raja and Muthukumar (2020). A specific dose of Spirulina contains a maximum amount of essential amino acids and vitamins, which determine the specificity of various metabolic activities in silkworms. Venkatesh *et al*. (2009) and Soliman (2021). Also, Kumar *et al*. (2019) reported that silkworms reared with an enhanced diet had greater proficiency in the transformation of ingested and processed food. Chlorella (*Chlorella vulgaris*) is a naturally single-celled green microalga that is known for its high protein content and abundance of vitamins, minerals, and chlorophyll. Furthermore, its considered a source of essential nutrients and has a very large percentage of essential and important amino acids, other nonessential acids, and many minerals, such as potassium, zinc, sulfur, calcium, magnesium and iron, in addition to proteins and many vitamins, such as vitamin E, vitamins A, B, and C. It is rich in vitamin B12 and chloroplasts and contains porphyrin, a substance that activates cellular metabolism. Thangaraj *et al*. (2022). Research suggests that Chlorella is rich in macro and micronutrients such as proteins, omega-3 fatty acids, polysaccharides, vitamins, and minerals, so Chlorella supplementation may help reduce oxidative stress, and It is believed to support detoxification, improve digestion, and enhance overall well-being, so chlorella is commonly used in dietary supplements and as a food ingredient. Coronado *et al*. (2023); Barghchi *et al*. (2023). Azolla (*Azolla caroliniana*) is a heterosporous aquatic fern that is a protein source and contains vitamins (B12, vitamin A and beta carotene), biopolymers, amino acids and minerals. Azolla is primarily used in agriculture as a green manure and biofertilizer Radhakrishnan *et al*. (2017). Many studies have shown the importance of Azolla supplementation on the economic traits of silkworms Airani *et al*. (2016); Moustafa (2024); Simon *et al*. (2024). In the view of the above-mentioned facts, this study was aimed to highlight the effects of non-traditional and sustainable protein sources such as Spirulina, Azolla and Chlorella on various silkworm characteristics, in order to assess their nutritional potential as supplementary feed for silkworms. This study is a valuable resource for farmers seeking to maximize profit when sustainable and unconventional protein is used in the rearing of silkworm and potentially leading to a more sustainable silk production process. Due to its promising potential, algae or fern meals could be a key factor for providing the nutritional requirements of silkworms and increasing the profitability of sericulture.

MATERIALS AND METHODS

The experiment was carried out at the Laboratory of Sericulture, Plant Protection Department, Faculty of Agriculture, South Valley University, Qena, Egypt. During spring 2024.

A-Preparation of Treatments:

- ***Spirulina platensis*** was procured from the Department of Botany, Fac. Science, South Valley University, Qena, Egypt.
- ***Chlorella vulgaris*** was procured from the Soil, Water and Environment Research Institute, Agriculture Research Centre. Giza, Egypt**.**
- ***Azolla caroliniana*** was procured from the farm of the Department of Agricultural Botany, Fac. Agriculture, South Valley University, Qena, Egypt.

B-Rearing of Silkworms

Local silkworm hybrids were obtained from the Sericulture Research Department, Plants Protection Institute, Agricultural Research Centre, Giza, Egypt. Then incubated at 24 ± 1°C and 93 ± 2% RH until hatching. Rearing was carried out under hygienic conditions,

according to Krishnaswami (1978). Mulberry leaves of *M. alba* var. Kanva-2 were used. Fourth-instar larvae were divided into four experimental groups, including the control group, with each group consisting of 150 larvae. Three replications were performed for each of the treatments. The powders (algae or fern) which used for the treatments were dusted on clean fresh mulberry leaves via fine mesh at a ratio of 1:15 w/w (powder/leaf). Additionally, the meal was given to the larvae one time a day during the 4^{th} and 5^{th} instar larvae.

C-Experimental Design:

Experimental rearing was carried out with uniformly sized larvae recruited from the stock that had been reared and acclimatized. These larvae were formed in triplicate and placed separately in the rearing trays. This rearing involved basic observations of the growth of larvae (final larval weight and silk gland weight), cocoon characteristics (cocoon, pupa and shell weights and Cocoon shell ratio %), moth fecundity (moth weight, ovary weight and number of laid eggs) and silk thread characteristics (thread weight, raw silk ratio and fibroin % and sericin %) of silkworms in response to the supplementation.

Statistical Analysis:

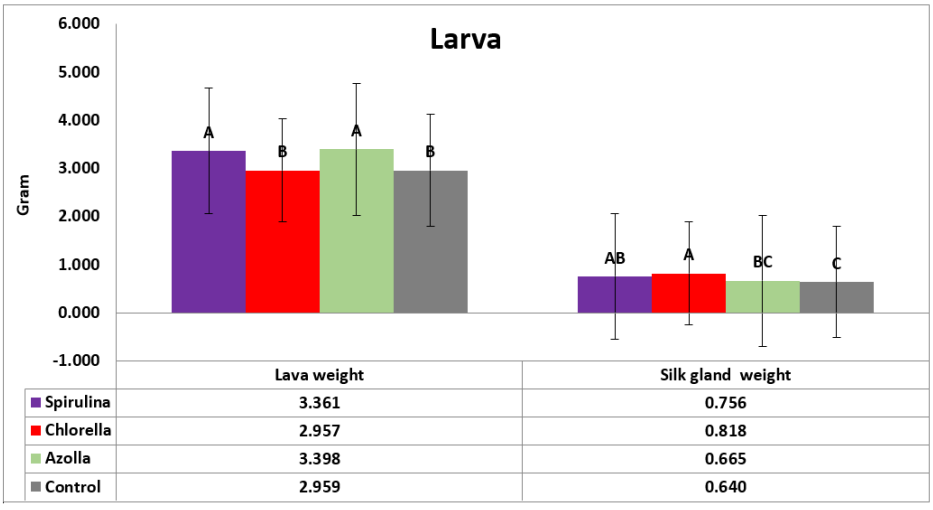
The obtained data were analysed via one-way analysis of variance (ANOVA) followed by Duncan's multiple range test via commercially available statistical SPSS software (SPSS Inc., 1999). The results are presented as the Means±SDs. $P \leq 0.01$ were regarded as statistically significant.

RESULTS AND DISCUSSION

Our data indicate that dietary treatments involving the use of sustainable protein sources, such as Spirulina, Chlorella and Azolla influence the biological, economical and technological characteristics of silkworms as follows:

Effects of Probiotics on Several Biological Characteristics of Silkworms:

The results revealed high significant differences in the 5^{th} instar larval weights between the treatment groups and the control group, the results were further visualized in (Fig. 1). The highest final larval weight was found in the Azolla group (3.398 g), whereas the highest silk gland weight was recorded in the Chlorella group (9.818 g). All the treatments resulted in greater increases than the control, possibly because of some of the biochemical processes involved in protein synthesis. The quantity and quality of dietary protein have long been considered important in the growth of silkworms. Hassan *et al*. (2020).



	Lava weight	Silk gland weight
Spirulina	3.361	0.756
Chlorella	2.957	0.818
Azolla	3.398	0.665
Control	2.959	0.640

Fig. 1: Effects of probiotics on larva characteristics of *Bombyx mori* L.
(Means followed by the same letter (s), in each axis, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT)).

Fecundity is the main factor in silkworm reproduction. We calculated the fertilization rate by counting and recording the number of laid eggs and weighting the ovaries and moths weights, which demonstrated the effects of these sustainable proteins on the fecundity of silkworms. There was no significant difference in the number of eggs laid between the experimental groups and the control group (Table 1) and illustrated at (Fig. 2). However, there was a significant difference in the moth and ovary weights between the experimental and control groups. The results revealed that Azolla regulates female fecundity by increasing moth weight, ovary weight and egg laying. This result indicates that mulberry leaves with Azolla have better outcomes in all stages of silkworm (larva and moth) development. The larval parameters were strongly influenced by the nutritive contents of mulberry leaves and the supplements. Compared with the control, mulberry leaves supplemented with Azolla presented greater larval weight and moth fecundity, possibly because Azolla is a rich source of protein (25–30%), essential amino acids (7–10%) and minerals (10–15%) Vijaykumar *et al.* (2016); Abdullah *et al.* (2024) and Kaur *et al.* (2024). Moreover, probiotics affect the physiology of digestion and various metabolic activities resulting in improvements in the growth and development of silkworm larvae as well as the maturation of silkmoths. Also, Simon *et al.* (2024). Feeding silkworms a diet rich in essential nutrients resulted in observable consequences for their development across different life stages. Borah and Boro (2020). Moustafa, (2024) informed that Azolla was the most effective treatment, positively impacting female fecundity and increasing the shell ratio in males. Additionally, it shortened the feeding period of the fifth larval stage, a crucial economic factor.

Table 1: Effects of probiotics on some biological characteristics of *B. mori*

Treatments	Final larval weight (g)	Silk gland weight (g)	Moth weight (g)	Ovary weight(g)	No.eggs
Spirulina	3.361±.17a	0.756±.08ab	0.765±.15a	0.509±.05a	261.33±17.5a
Chlorella	2.957±.41b	0.818±.05a	0.743±.11a	0.451±.04ab	313.67±90.69a
Azolla	3.398±.13a	0.665±.04bc	0.876±.05a	0.51±.05a	361.67±78.69a
Control	2.959±.35b	0.64±.09c	0.547±.05b	0.412±.01c	249.33±81.99a

(Values are the mean of the observations ± standard deviation (SD). Means followed by the same letter (s), in each column, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT)).

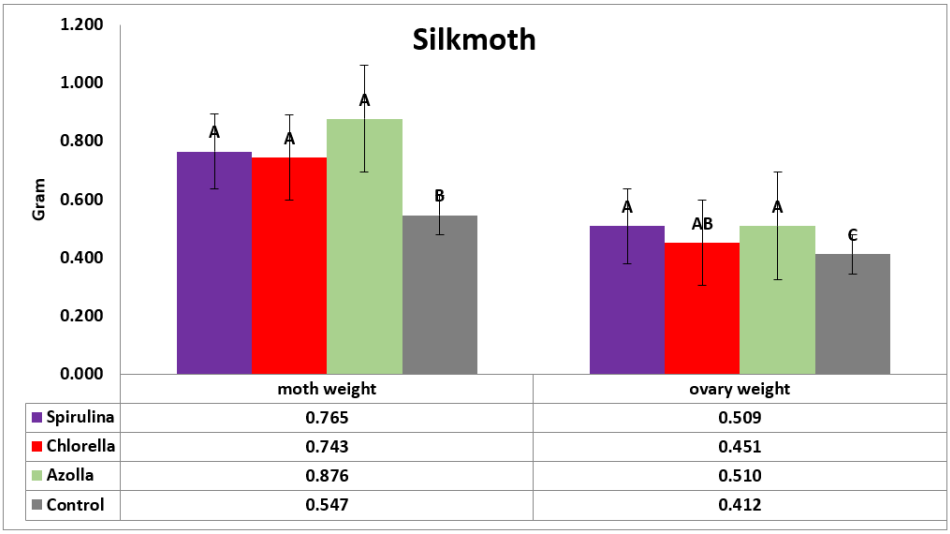


Fig. 2: Effects of probiotics on moth characteristics of *Bombyx mori* L.
(Means followed by the same letter (s), in each axis, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT)).

Effects of Probiotics on Several Economic Characteristics of Silkworms:

The cocoon shell weight and cocoon shell rate can reflect cocoon quality and silk production in the sericulture industry. Therefore, after probiotic supplementation, we investigated cocoon production on day 7 after cocooning. Analysis of variance revealed highly significant differences ($p \leq 0.01$) between the treatment values with respect to pupal weight and the cocoon shell ratio as showed in (Table 2) and illustrated at (Fig. 3). Compared with the control treatment, Azolla treatment resulted in very greater increases in the single cocoon weight and pupal weight, and compared with the control treatment, the Chlorella treatment had a greater effect on the single shell weight and cocoon shell ratio.

Table 2: Effects of probiotics on some economical characteristics of *B. mori*

Treatments	Fresh cocoon weight (g)	Pupal weight (g)	Cocoon shell weight (g)	Cocoon shell ratio %
Spirulina	1.019±.05 ab	0.789±.05 ab	0.23±.02 ab	22.658±1.95 ab
Chlorella	0.956±.05 b	0.694±.05 b	0.261±.04 a	27.338±4.54a
Azolla	1.123±.08 a	0.898±.08 a	0.225±.01 ab	20.068±.1.81bc
Control	1.071±.08 ab	0.893±.05 a	0.178±.02 b	16.569±.9c

(Values are the mean of the observations ± standard deviation (SD). Means followed by the same letter (s), in each column, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT)).

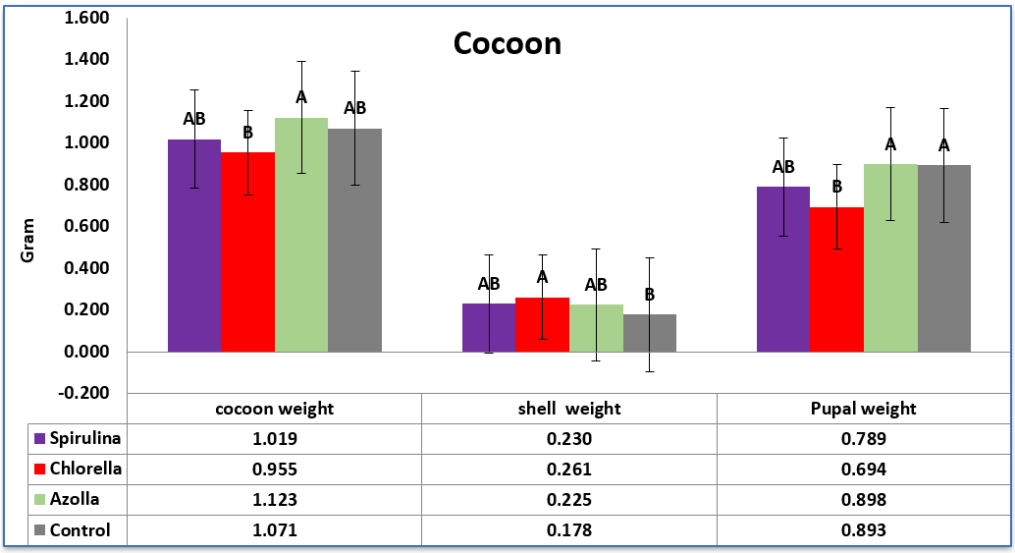


Fig. 3: Effects of probiotics on cocoons characteristics of *Bombyx mori* L.
(Means followed by the same letter (s), in each axis, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT))

The cocoon weight, shell weight, shell ratio, and silk production ratio depend on the rearing conditions, quality, nutritive elements and quantity of mulberry leaves supplied during rearing, and a relatively high weight indicates the approximate quantity of raw silk output. Mulberry leaves constitute the sole food plant for the silkworm. The addition of mulberry leaves from proteinaceous sources can increase cocoon and shell weights and improve silk production. This is attributed to the assumption that the nutritive requirements of the larvae might have been fulfilled because of feed supplementation rather than unenriched mulberry leaves. (Raja and Kumar 2016 and Vijaykumar *et al*. 2016). In light of the above view, it is very clear that there is appreciable improvement in silk production. Additionally, various investigations have shown that Chlorella has high nutritional value and it's a safe source of protein for consumption and that dietary supplementation with Chlorella

may increase growth; economic traits; silk gland ratio; and the biochemical protein, carbohydrate and lipid contents of the silk gland, heamolymph, fat body and muscles. (Selin and Raja 2021; Mirzayeva and Nurlimuratova, 2023 and Panaite *et al*. 2023).

Effects of Probiotics on Several Technological Characteristics of Silkworms:

In the present study, the results revealed that the feeding efficiency of Azolla had a more pronounced effect on increasing thread weight than Chlorella or Spirulina supplemented diets. The weights of threads in all experimental groups were greater than those in the control group. The results are illustrated in (Table 3 and Fig. 4).

Table 3: Effects of probiotics on some technological characteristics of *B. mori*

Treatments	Thread weight (g)	Raw silk ratio %	Fibroin %	Sericin %
Spirulina	0.215±.02 a	52.33±.89 a	68.23±5.32 ab	31.77±5.32 ab
Chlorella	0.207±.01 a	53.62±2.91 a	74.22±2.11 a	25.78±2.11 b
Azolla	0.229±±.01 a	53.56±2.89 a	67.84±4.52 ab	32.16±4.52 ab
Control	0.205±±.01 a	50.2±1.33 a	65.38±2.21 b	34.62±2.21 a

(Values are the mean of the observations ± standard deviation (SD). Means followed by the same letter (s), in each column, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT)).

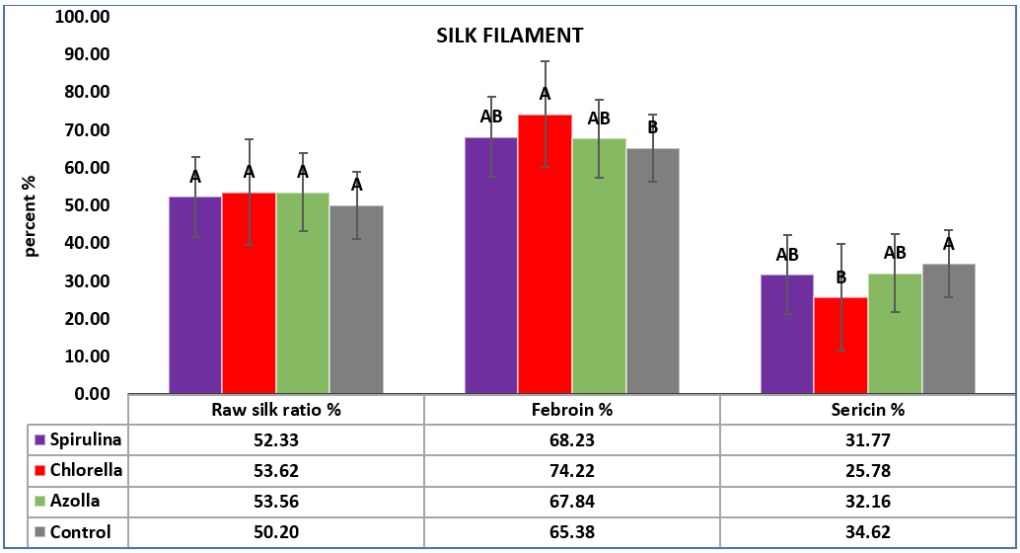


Fig. 4: Effects of probiotics on silk filament characteristics of *Bombyx mori* L.
(Means followed by the same letter (s), in each axis, are not significantly different at 0.05 level probability, by Duncan's multiple range test (DRMT)).

The highest percentage increase in raw silk was observed in the Chlorella treatment group (50.2%). The amount of fibroin was highest in the Chlorella group (74.22%), followed by Spirulina (68.23%) and Azolla (67.84%), compared to the control group (65.38%). In contrast, the control group showed the highest increase in sericin content (34.62%). Overall, all probiotics had positive impacts on all technological characteristics. The present study is aligning with findings of Raja and Kumar (2016), who demonstrated that silkworm diets supplemented with extracts of Chlorella and Scenedesmus algae can improve growth and economic traits. In their study, the economic traits of silkworms, such as cocoon weight, pupal weight, shell weight, shell ratio, filament length, fibroin content, and denier, were enhanced by algal supplementation. These results suggest that algal supplementation can be a beneficial practice for farmers to increase both the quality and quantity of silk yield. Also,

Murugan *et al*. (1998) noticed a strong correlation between the growth and silk production of silkworms treated with plant extracts. They attributed the growth-promoting effect of the plant extracts to the stimulation of biochemical processes leading to protein synthesis.

The effect of Azolla was consistent across most characteristics, except for silk gland weight, shell weight, cocoon-shell ratio, raw silk percentage, and fibroin percentage, where the Chlorella group showed superior performance. Therefore, Chlorella treatment had a strong impact on cocoon characteristics, which is reflected in the quality and quantity of raw silk output. The results of the present study indicate that, compared to Azolla and Spirulina, Chlorella treatment significantly increased silk production. The beneficial effects of Chlorella may be attributed to the synergistic interactions of its various nutrients and antioxidants. The results of the present study are consistent with those of Mirzayeva and Nurlimuratova (2023), who reported that water-soluble proteins and vitamins (B2, B6, and C) in Chlorella microalgae can enhance silkworm growth, disease resistance, and productivity. Larval diet is considered one of the primary factors determining the abundance of immune cells, as demonstrated by Gogoi *et al.* (2022). Therefore, the results of this study recommend adding Chlorella as a feed supplement to silkworms to effectively increase silk gland weight, shell weight, raw silk percentage, and fibroin content. In general, the addition of these sustainable protein sources, rich in essential amino acids and vitamins, can enhance various metabolic activities in silkworms and increase silk production.

Conclusion

The present study investigated the impact of sustainable protein sources such as Chlorella, Spirulina and Azolla on various characteristics of *B. mori*. Although Azolla was the most effective treatment overall, Chlorella was the most recommended treatment due to its positive impact on several key parameters. These parameters, including silk gland weight, shell weight, cocoon-shell ratio, raw silk percentage, and fibroin content, are crucial for silk quality and quantity. The results revealed that increasing the nutrient quality of mulberry leaves through the addition of sustainable supplements such as algae and ferns enhanced larval and moth productivity, improved silk production, and may have a positive impact on the entire sericulture industry.

Declarations

Ethical Approval: Ethical Approval is not applicable.

Competing interests: The authors declare no conflict of interest.

Funding: No funding was received.

Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

Acknowledgements: Not applicable.

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