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Effect on the Probionts to the Enhancement of Silk Proteins (Sericin and Fibroin) in the Silk Gland and Cocoons of Silkworm (*L× CSR2*) *Bombyx Mori* (L.)

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Abstracts: *The domesticated silkworm, Bombyx mori (L), a Lepidopteran molecular model and an important economic insect. The silkworm, B. mori produces massive amount of silk proteins during the final stage of larval development (Fifth instars). These proteins are stored in the middle silk gland and they are discharged through the anterior duct and spinneret, at the end of the fifth instar. There two kinds of silk proteins have been distinguished as major components of silk cocoons, the first being real silk fibre namely fibroin (H-chain), and the second being adhesive substance namely sericin (L-chain). The silkworm is being used as biofactory for the production in the silk industry, which has promoted by the application of five probiotic supplementation feeding techniques. The probiotic candidates were identified and used in the nutritional augmentation and the production of quantitatively and qualitatively improved silk. The outcome this research will be a boon to the silk farmers.*

Keywords: *Probionts, Silk Gland, Cocoon, Silk Proteins, SDS-PAGE.*

I. Introduction

Nutritional status and environmental conditions play a vital role for the development of silkworm, *Bombyx mori*. Main sources of metabolic fuels are proteins the building blocks and energy reserves. Proteins are required in all the stages and particularly during the fifth larval stage of *Bombyx mori*. A higher quantity of protein is essential for the formation of sericin and fibroin during spinning of silk cocoons. Growth and development of the larvae and subsequent cocoon production are very much influenced by the nutritive value of mulberry leaves [1], [2]. Growth of the silkworm during metamorphosis is characterized by increase in bodyweight, and accumulation and transport of various biochemical constituents like proteins, amino acids, carbohydrates, lipids and some enzymes [3].

Quantitative changes in the storage proteins during the larval development of silkworm have also been observed by Nagata and Kobayashi [4]. Choudhuri and Medda [5] conducted research in the fat body of female *Bombyx mori* and confirmed its role in glycogen synthesis. Much of the biochemical studies in silk glands have been centered around the synthesis of the silk proteins, fibroin and sericin. Fibroin and sericin are secreted in the fourth and fifth instar stages of larval metamorphosis. The posterior silk gland is the seat of synthesis of fibroin, while the middle region of the gland secretes sericin [6]. Anterior region of the silk gland does not seem to have any secreting function, but acts as a passage that carries the silk substance from the reservoir of the middle region.

Importance of research on the effect of mulberry fortification agents in silkworm nutrition can be judged from the principle of co-operating supplements [7]. Supplementary nutrients are chemicals which, when added to normal food increase the nutritional value of the food, making it more useful [8]. Earlier research has demonstrated the effect of feed supplements on silkworm growth and silk production. However, research on the synergic action of a combination of supplementary nutrients is meagre. Various researches have been carried out on the bacterial supplementation of mulberry leaves which are fed to silkworms. In the present study, beneficial bacteria of silkworms were tried as feed supplement to the host. These bacteria are referred to as probiotics, (live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance). There are five bacterial strains were shown that the gut of *B. mori* can harbour a variety of bacteria, of which five species were dominant, non-pathogenic and with high growth, enzyme potential and on the biochemistry of silkworms has been studied.

II. Material and Methods

Experimental Protocol

Field study was carried out during Jan - April 2016 at the Maruthai's Seri-farm at Othakadai, Tiruchirapalli district. The advanced shelf (Rack) rearing method suggested by Krishnaswami *et al.,* [9] was followed. Leaves of 55-65 day old shoots were ideal for the post larval rearing. Whole shoot or branches with mulberry leaves were used for feeding the silkworms. Larvae, on reaching the third instar, immediately after the second moult, were separated in to seven groups: namely six experimental groups and one control group.

Physical Parameters

To maintain in the optimum temperature was maintained approximately at 24 - 26°C, humidity was 70 - 80%, air current (1.0m/second and the photoperiod also maintained at 16 hours light and 8 hours darkness) respectively for the third, fourth and fifth instar.

Probiotic Feed Supplementation

In the present experiment, the following putative probiotic strains: *Bacillus cereus, Bacillus subtilis, Bacillus amyloliquefaciens, Lactobacillus casei, Lactobacillus plantarum* and mixed bacteria with soy flour were sprayed on mulberry leaves for fortification.

Mulberry Fortification

Prior to feeding, the freshly prepared bacterial cells of each strain were re-suspended in cool saline (0.9%) solution. For the third instar larvae, the probiotic stock solution was prepared in the ratio of 15:1000 ml, (i.e. 15 ml stock bacterial solution suspended in 1000 ml of sterile saline water). 10:1000 ml dilution was done for the feeding of fourth instar larvae and 5:1000 ml for the early fifth instar and late fifth instar larvae. Each suspension was sprayed on the freshly plucked mulberry leaves in a uniform spread. For mixed bacterial-diet, equal proportions of each bacterial strain and soyabean flour were mixed and diluted with appropriate amount of sterile saline.

Biochemical Analysis

Biochemical estimations such as protein [10], carbohydrate (Anthrone method), lipids [11] and sericin and fibroin content of the cocoons was carried out [12], the protein profiles [13] were carried out in the silk gland and the extract of the cocoons.

Sericin and Fibroin Content

Initial dry weight of the shell (mg) - Dry weight (mg) of the shell after alkali treatment = Sericin (gum spot) content in mg/shell or cocoon. Dry weight of the shell (mg) - Sericin content (mg) = Fibroin (silk fibre) content (mg/shell or cocoon).

III. Results

In the present study the growth promoting properties of the chosen bacteria: *Bacillus cereus, Bacillus subtilis, Bacillus amyloliquefaciens, Lactobacillus casei, Lactobacillus plantarum* and a combined diet was assessed in *Bombyx mori* by coating mulberry leaves with the bacteria and feeding the silkworms.

Protein Content of Silk Gland

Protein content of the silk gland was 1.436mg/100mg in the normally fed third instar larvae. As the larvae grew, the protein content in the silk gland also increased, and in the late fifth instar, the silk gland had 3.326mg/100mg protein (Table 1).

Table 1- Biochemical contents of Protein, Carbohydrate and Lipids in the body tissues of silk gland of *B. mori* fed on mulberry leaves supplemented with putative probiotic strains

S.No.	Silk Glands (mg/100 mg)																				
	Protein content							Carbohydrate content							Fat content						
Instars	Con	A	B	C	D	E	F	Con	A	B	C	D	E	F	Con	A	B	C	D	E	F
III instar	1.4368	1.4330	1.3398	1.6058	1.6542	1.4174	1.6542	0.0343	0.0258	0.0174	0.0258	0.0300	0.0250	0.0266	0.017	0.019	0.010	0.021	0.026	0.017	0.022
IV instar	2.2892	2.5164	1.9028	2.6700	2.4540	2.0620	2.5514	0.0434	0.0552	0.0326	0.0584	0.0484	0.0426	0.0476	0.030	0.042	0.024	0.039	0.028	0.024	0.036
Early V instar	3.2600	2.3262	1.9746	3.3280	3.3786	3.1320	3.2620	0.0484	0.0618	0.0358	0.0802	0.0476	0.0434	0.0534	0.050	0.047	0.029	0.056	0.039	0.032	0.044
Late V instar	3.3262	3.3902	2.1864	3.6174	3.4678	3.2892	3.5728	0.0108	0.0158	0.0074	0.0234	0.0158	0.0082	0.0166	0.031	0.027	0.023	0.042	0.031	0.026	0.032

Con : Control Strain - A : *B. cereus* Strain - B : *B.subtilis* Strain – C : *B. amyloliquefaciens*
Strain - D : *L. casei* Strain - E : *L. plantarum* Strain - F : Combined bacterial diet with soy flour

In general, bacterial supplementation to the mulberry leaves had a positive effect on the protein content of the silk gland. Third instar larvae fed on *B. amyloliquefaciens* coated leaves had a silk gland-protein content of 1.6058 mg/100 mg, while third instar larvae fed similarly with *L. casei* and combined bacterial diet had 1.654mg/100mg protein in their silk gland (Fig. 1). However supplementation with *B. subtilis* and *L. plantarum* had lesser effect on the protein content in the silk gland. As observed in normal leaf fed control larvae, the experimental groups also registered a progressive change in the silk gland protein with the progression of the instar stages). This increase was maximum 3.617mg/100mg for *B. amyloliquefaciens* supplemented larvae, followed by combined bacterial supplemented larvae (3.572 mg/100 mg) and *L. casei* supplemented ones (3.467 mg/100 mg). *B. cereus* supplementation created an effect closer to that in control group although with a steep decline during the early fifth instar and subsequent revival. However feeding the larvae with leaves coated with *L. plantarum* had a slightly lesser effect on the silk gland protein augmentation, while this effect was noticeably retrogressive in the larvae fed on *B. subtilis* coated leaves.

Fig. 1 Protein content (mg / 100 mg) in the silk gland of *Bombyx mori* fed on mulberry leaves supplemented with putative probiotic strains

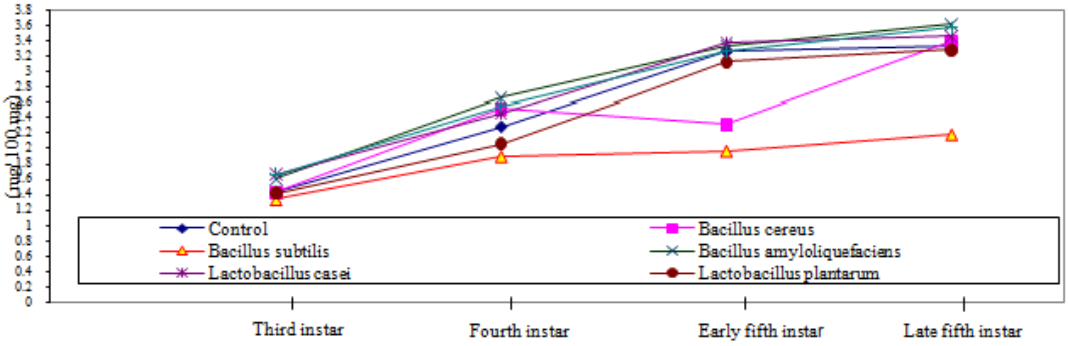


Fig. 2 Carbohydrate content (mg / 100 mg) in the silk gland of *Bombyx mori* fed on mulberry leaves supplemented with putative probiotic strains

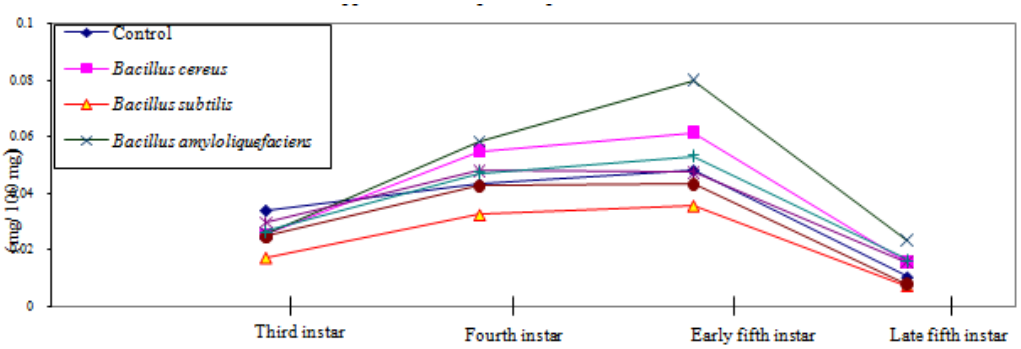
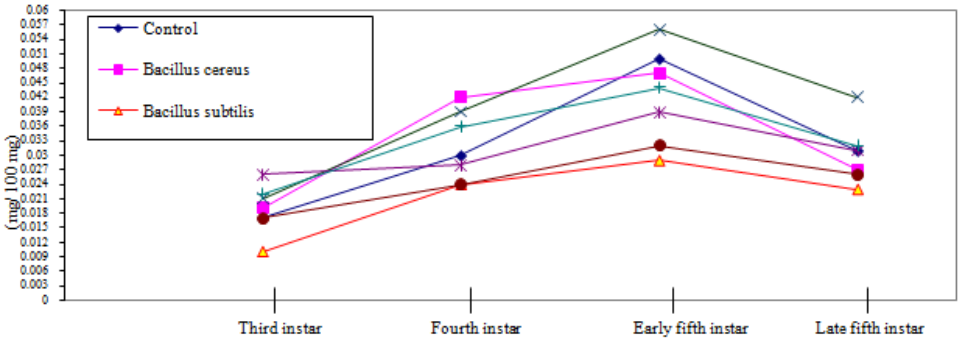


Fig. 3 Lipid content (mg / 100 mg) in the silk gland of *Bombyx mori* fed on mulberry leaves supplemented with putative probiotic strains



Carbohydrate content of silk gland

Carbohydrates in general were at higher levels in *B. mori* silk gland than the other tissues. Normally fed third instar larval silk gland (control) and experimental groups had carbohydrates in the range: 0.0174 - 0.0343 mg/100mg

(Table-1). Carbohydrates of silk gland gradually increased up to the early fifth instar stage, beyond which there was a sudden decline (Fig. 1.2). This increase was at the highest rate in larvae supplemented with *B. amyloliquefaciens, B. cereus* and combined bacterial diet, while in those fed on *B. subtilis and L. plantarum* the increase was at a lesser rate than the control larvae.

Lipid Content of the Silk Gland

Among the various feed groups, the silk gland lipids varied between 0.017 to 0.026 mg/100 mg at the third instar stage (Table - 1). In all the feed groups, the silk gland lipids increased steadily till the early fifth instar stage and thereafter, there was a gradual fall. The increase in the silk gland lipids was the highest in the control and *B. amyloliquefaciens* supplemented early fifth instar larvae (Fig. 3). Early fifth instar lipids of silk glands for the other feed-groups were in the following receding order: *B. cereus* → combined bacterial diet → *L. casei* → *L. plantarum* → *B. subtilis.*

Sericin and Fibroin Content of Cocoons

The sercin content (gum spot) of the cocoons produced by the variedly fed larvae was more or less similar, while the fibroin content varied (Table - 2). Maximum fibroin content of 0.310 g could be observed in the cocoons from *B. amyloliquefaciens* supplemented larvae, followed by those from *B. cereus* supplemented ones (0.270 g).

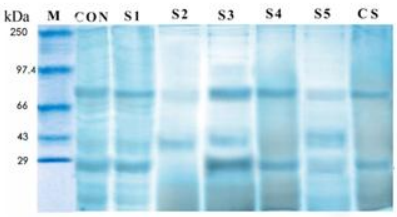
Table 2 - Sericin and fibroin (protein) content in the cocoons fed on V-1 mulberry leaves supplemented with putative probiotic strains

Types of probiotic strains	Shells		
	Initial weight (g)	Sericin content- gum spot (g)	Fibroin content- real silk (g)
Control	0.23	0.046	0.184
B. cereus	0.35	0.080	0.270
B. subtilis	0.23	0.046	0.184
B. amyloliquefaciens	0.40	0.090	0.310
L. casei	0.28	0.056	0.224
L. plantarum	0.31	0.068	0.242
Combined bacterial diet with soy flour	0.34	0.070	0.270

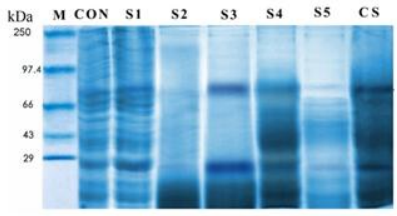
Sericin content: Initial weight of the shell - final weight of the shell after the alkaline treatment
Fibroin content: Initial weight of the shell - Sericin content

SDS – PAGE Electrophoresis of the Silk Gland

Plate 1.1

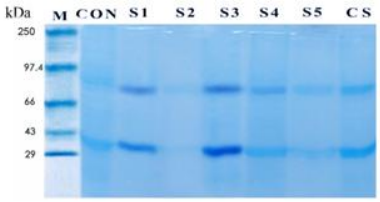


1.1 a Electrophorogram (SDS-PAGE) of protein in the third instar silk gland of silkworm (*Bombyx mori*) fed on different probiotic coated V-1 mulberry leaves

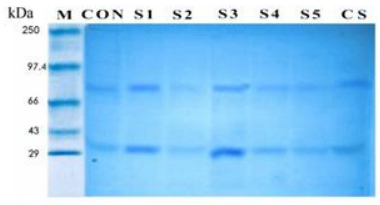


1.1b Electrophorogram (SDS-PAGE) of protein in the fourth instar silk gland of silkworm (*Bombyx mori*) fed on different probiotic coated V-1 mulberry leaves

Plate 1.2



1.2 a Electrophorogram (SDS-PAGE) of protein in the early fifth instar silk gland of silkworm (*Bombyx mori*) fed on different probiotic coated V-1 mulberry leaves



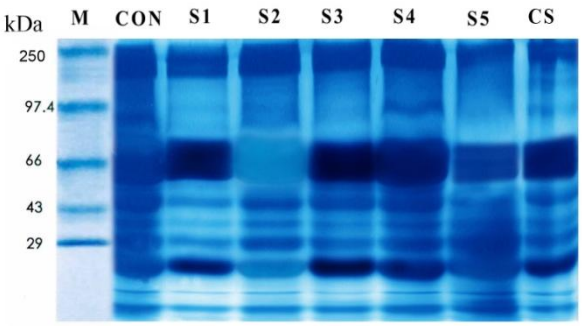
1.2 b Electrophorogram (SDS-PAGE) of protein in the late fifth instar silk gland of silkworm (*Bombyx mori*) fed on different probiotic coated V-1 mulberry leaves

Several polypeptide fractions could be observed in the silk glands of control (Lane CON) and *B. cereus* supplemented third instar larvae (Plate 1.1a,b). The 30 kDa and 80 kDa fractions were particularly dense and prominent in all the feed groups except *B. subtilis* (Lane S2) and *L. plantarum* (Lane S5) supplemented ones. Although there were new bands appearing, those at 30kDa and 80kDa remained distinct in the fourth instar larvae also (Plate 1.1b), with the

exception of lane S2, which represented the *B. subtilis* supplemented larvae. In *L. plantarum* supplemented larvae (Lane S5), feeble representations of these fractions were evident. In the early and late fifth instar stage the silk gland could show only two major polypeptide fractions, one at the 30 kDa region and the other at 80kDa region (Plates 1.2a, 1.2b). The bands were feeble in *B. subtilis* fed larvae but very prominent in *B. cereus* and *B. amyloliquefaciens* supplemented larvae.

SDS-PAGE Electrophoresis of the Cocoons

Plate – 1.3



Electrophorogram (SDS-PAGE) of protein in the cocoons of silkworm (*Bombyx mori*) fed on different probiotic coated mulberry leaves

Proteins of the cocoons produced by *B. mori* fed on various bacteria-coated mulberry leaves were electrophoresed and the electrophorogram is presented in Plate 1.3. Electrophoretic separation of the proteins was hampered to a great extent, probably because of the heavy concentration of proteins in the cocoon and their complex nature. All the experimental feed-groups showed 3 distinct, dense bands: one at 30 kDa regions, the second at 66 kDa regions and the third at 150 – 205 kDa regions. Bands at 80 kDa were not as distinct as of the silk gland or fat body. However, several distinct polypeptide fractions could be observed at regions lower than 66 kDa molecular weight, some even at lesser than the 29 kDa region.

IV. Discussion

Mulberry (*Morus* species) leaf is the energy rich food and source of nutrition for the silkworm, *Bombyx mori L*. The growth and development of larvae, and subsequent cocoon production are greatly influenced by nutritional quality of mulberry leaves. In recent years attempts have been made in sericulture to use nutrient supplements such as proteins, carbohydrates, amino acids, vitamins, sterols, hormones, antibiotics etc, for better performance and to get higher yield of quality of cocoons [14]. The silk fiber protein is synthesized by silk gland cells and stored in the lumen of the silk glands. Subsequently, it is converted into silk fibres. When the silkworms secrete the liquid silk during the spinning, it passes through the anterior gland and expelled out through the spinneret opening [15]. The carbohydrates protein and lipids bimolecules are supplied by feeding on mulberry leaves. Although the mulberry leaves are complete diet for silkworm it is possible that some deficiencies occur for different reasons [16].

Nutritional contributions and the symbiotic benefits offered by insect gut-dwelling bacteria [17], [18] is an area which can substantially modify and promote the health and silk production capacity of *B. mori*, although this field has only attained limited attention in the sericulture scenario. Live bacterial preparations called probiotics are usually used feed supplementation towards the improvement of biochemical parameters like protein, CHO and lipids. Different species of lactic acid bacteria have been extensively studied [19] are found to be beneficial as probiotics [20], [21]. In the present study new probiotic candidates: *B. cereus, B. subtilis, B. amyloliquefaciens, L. casei, L. plantarum* and a combined *bacterial* diet were selected, because of their perceived colonization ability in the gut of *B. mori*.

The probiotic *Lactobacillus* have been reported to improve the cocoon production of mulberry silkworm *Bombyx mori* [22]. Certain probiotic bacteria not only enhance the probiont efficiency but also inhibit the growth of pathogenic microbes. *Streptomyces noursei* are considered probiotic because of their antibacterial activity and its rolein the ecofriendly management of silkworm diseases [23]. Kamioka *et al*., [24] observed that the cocoon quality could be improved to an extent when mulberry leaves were coated with soyabean flour. Nirmala *et al.,* [25] showed that protease activity increased in the gut of *Bombyx mori* larvae reared on soy protein. Similar results were obtained by supplementation of different nutrients including proteins [26], [27].

Among the selected bacteria, *B. amyloliquefaciens* contributed more towards the efficiency of digestion and assimilation of food materials, leading to increased protein synthesis and subsequent accumulation of storage proteins

in the body of host. Besides, *B. amyloliquefaciens* might have contributed to the protein content of the feed in the present study. Beneficial effects by modulation of gut micro-flora and their influence on mucosal immunity or through altering enzymatic activities has been extensively studied in man, animals and many insects [28]. From the results of the present investigation, it could be inferred that when the feeding behavior was more pronounced in the silk gland and cocoons.

A detailed analysis on the interrelationships between silk gland and other body tissues such as fat body and haemolymph of fifth instar *B. mori* was done by Noguchi *et al.,* [29]. Although several polypeptide fractions were discernible in silk glands, most of them were feeble, except those at 30 kDa and 80 kDa regions. The 30 kDa and 80 kDa bands were prominent in both the tissues as the metamorphosis progressed. Besides, the fat bodies, presumed to be the equivalent of vertebrate liver, showed several other fractions of low and high molecular weights under the nutritional influence of supplemented bacteria, throughout the V^{th} instar period. Presence of five protein bands, from 6 kDa to 67 kDa was reported by Lokesh and Ananthanarayana [30] in silk worms exposed to a mutagen, DES.

Among the polypeptide fractions, 30 kDa and 80 kDa fractions were common in the tissue; silk gland. These fractions became denser as the metamorphosis progressed. The presence of dense 30 kDa levels in the V^{th} instar silk gland and a decrease beyond pupation was reported Pushpa and Gopinathan [31]. Park *et al*., [32] were of the opinion that 30 kDa proteins are involved in larval lipid transport. The dominance of 80 kDa till the pupation in the silk gland and the prominence of 30 kDa during the early V^{th} instar in the haemolymph, and its disappearance during exclusion were reported by Izumi *et al*., [33]. Li *et al*., [34] did proteomic analysis of V^{th} instar *B.mori* and they could observe that 75% of the total polypeptide fractions (241) were between 35 – 90 kDa region and 57 new polypeptide fractions were expressed in the late V^{th} instar which could be related to the synthesis of silk proteins and the metamorphosis leading to pupation [35].

V. Conclusion

The present study on the growth biochemical constituents and electrophoretic spectrum of the tissues (Silk gland) thus prove the enhancing effect of the putative probionts: *Bacillus amyloliquefaciens Bacillus cereus, Lactobacillus casei* and a bacterial consortium on the physiology, metamorphosis and silk production in *Bombyx mori*.

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