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Impact of probiotics on enzymatic activity and economic traits of double hybrid silkworm, *Bombyx mori* L

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Abstract
Mulberry nutrition plays an important role in quality silk production. An investigation was made to study the supplementation of probiotics to silkworm larvae and its impact on enzymatic profile and economic parameters of double hybrid silkworm, *Bombyx mori*. Three types of probiotics *viz.*, *Lactobacillus rhamnosus, Sacchoromyces boulardii, and Bifidobacterium longum* and concentration ranged from 0.5 to 3.0 per cent were used for the study. Silkworm larvae were fed on mulberry leaves treated with 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3% concentrations of probiotics from 3rd instar onwards and its enzyme activity and economic traits *viz.*, cocoon weight (gm), pupal weight (gm), shell weight (gm) and shell ratio (%) were recorded. The results revealed that increase in concentration of probiotics showed increase in amylase and invertase activity. Among these probiotics, *Bifidobacterium longum* recorded maximum enzymatic activity and highest cocoon traits followed by *Lactobacillus rhamnosus* and *Saccharomyces boulardii*.

Keywords: probiotics, *Lactobacillus rhamnosus, Sacchoromyces boulardii, Bifidobacterium longum,* silkworm, economic parameters

1. Introduction
Probiotics are the preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora by implantation or colonization, in a compartment of the host and by that, exert beneficial effects on host health. Food and Agriculture Organization of the United Nations-World Health Organization (FAO-WHO) officially defined probiotics as "live microorganisms when administered in an adequate amounts confer a significant health benefit on the host" (FAO, 2001). These beneficial microorganisms are ingested and thereby introduced into the intestinal microflora intentionally and resulted in high numbers of beneficial bacteria to participate in competition for nutrients with and starving off harmful bacteria (Mombelli and Gismondo, 2000) [6]. The probiotics take part in many number of positive health promoting activities in human physiology. The beneficial effects of the ingested probiotic bacteria are provided by those organisms that adhere to the intestinal epithelium. The presence and adherence of probiotics to the mucous membrane of the intestines build up a strong natural biological barrier for many pathogenic bacteria (Yang *et al*., 2011) [12].

Silkworm, B. mori is monophagous and host plant-specific insect that feeds solely on mulberry leaves (*Morus* sp., Family: Moracea) produces silk. The growth and development of silkworm larvae and subsequent cocoon production are greatly influenced by the nutritional quality of mulberry leaves. In addition, nutritional supplements include vitamins, amino acids, protiens and probiotics when added to larval feed tend to increase nutritional efficiency and economic traits of silkworm. The products containing probiotic bacteria are widely explored, thereby increasing the importance of their accurate specializations and the beneficial effects by the activity of gut micro-flora and its influence on mucosal immunity through altering the enzymatic activities which has been extensively studied in human, animals, and many insects. No commercial probiotics formulations are specifically designed for sericulture though they are available for human, aquaculture and veterinary medicinal use. Few limited research works on use of probiotics for silkworm growth and development were reported. With this background, the present investigation was carried out to study the impact of probiotics on enzymatic profile and economic traits of double hybrid silkworm.

2. Materials and Methods

2.1 Enzymatic analysis

The fifth instar double hybrid larvae were collected randomly from the rearing bed and starved for 2 h and kept in a clean closed container containing dried tobacco leaves. The starved larvae were placed on the plastic paper over the leaves. After 20 minutes, the regurgitated digestive juice on the plastic paper was collected with syringes and samples were stored at – 20 ^{0}C to avoid oxidation. The digestive juice was centrifuged at 5000 rpm for 5 minutes in 4 ^{0}C to get clear solution, which was used for an enzyme analysis.

2.1.1. Quantitative assay of amylase

The starch digestive juice was used as the substrate and maltose was the end product. Dinitrosalicylic acid (DNSA) was added to give colour [reddish orange] and to stop the reaction of enzyme over the substrate. 10 µl of digestive juice sample was taken in test tubes, added with 2 ml of 0.2 per cent starch solution and incubated at 37 ^{0} C for 60 minutes. After that, 2 ml of DNSA solution was added to each tube and boiled on hot water bath for 5 minutes. Finally, the optical density (OD) values were measured at 525 nm. Serial dilutions were prepared from the stock solution of maltose from 100µl up to 2 ml to determine the concentrations of maltose. For each sample of 2 ml, 2 ml of DNSA was added and boiled for 5 minutes in water bath. After cooling, the OD values of the serially diluted maltose solutions were recorded at 525 nm. The standard graph was prepared by plotting the OD values against the serial dilutions. The amylase activity in sample was calculated using standard graph and it was expressed as µg /100 µl of digestive juice in 60 minutes.

2.1.2. Quantitative assay of invertase

The enzymatic assay of invertase was measured at pH 7.4 with the temperature of 37 ºC. Four test tubes were taken and to this 2.5 ml of sucrose solution and 0.25 ml of sample was added to each test tube and 2 ml of phosphate buffer was added to the above and incubated at 37ºc for 1 hour. Then immediately 0.5 ml of 4% NaOH along with 0.5 ml of DNS reagent was added and kept in boiling water bath for 10 minutes. Then after cooling OD was read at 500nm using Colorimetry. The replication was done in triplicates to get the final OD value (Jeyaraman, 1981) [3]. The enzyme levels were calculated using the formula described by Bernard and Prosser (1973) [1].

Enzyme calculation =OD of unknown x std. concentration x Dilution factor x 1 OD of known mg protein.

2.2. Supplementation of probiotics to silkworm larvae

Commercial formulation of probiotics ((10^6 cfu/ml) were fed to silkworm larvae through feed supplementation as per rearing methods suggested by Krishnaswami *et. al* (1978) [4]. Mulberry variety V1 and double hybrid silkworm was used for the study. Silkworm were fed with untreated leaves until the end of III instar stage. Freshly moulted IV instar larvae were divided into six groups for the treatment each group comprising of 30 larvae and one group served as a control. The freshly plucked mulberry leaves were washed with tap water and cleaned. The clean mulberry leaves were then completely dipped in six different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) of probiotics *viz.*, *Lactobacillus rhamnosus*, *Bifidobacterium longum* and *Saccharomyces boulardii* with control. The leaves were dipped in such a way that both the dorsal and ventral side of the leaves contained the probionts and the treated leaves were allowed to dry for 15 minutes. The leaves of the control worms were dipped in water and dried before feeding. Parameters like total cocoon weight (gm), pupal weight (gm), shell weight (gm) and shell ratio (%) were recorded. Experiment was conducted in CRD with each treatment replicated thrice and 30 larvae per replication were used.

3. Results and Discussion

3.1. Impact on enzymatic profile

The findings of the present study revealed that, when the probiotics *Lactobacillus rhamnosus*, *Sacchoromyces boulardii*, and *Bifidobacterium longum* used as supplementary feed, there was a profound increase in the enzymatic activity of amylase and invertase in the treated larvae when compared to control. Among the three probiotics, *Bifidobacterium longum* @ 3% showed highest amylase activity of 67.89 mg/g in double hybrid followed by *Saccharomyces boulardii* which showed 67.01 mg/g of activity and *Lactobacillus rhamnosus* of 28.32 mg/ g when compared to control (23.63 mg /g) (Fig. 1).

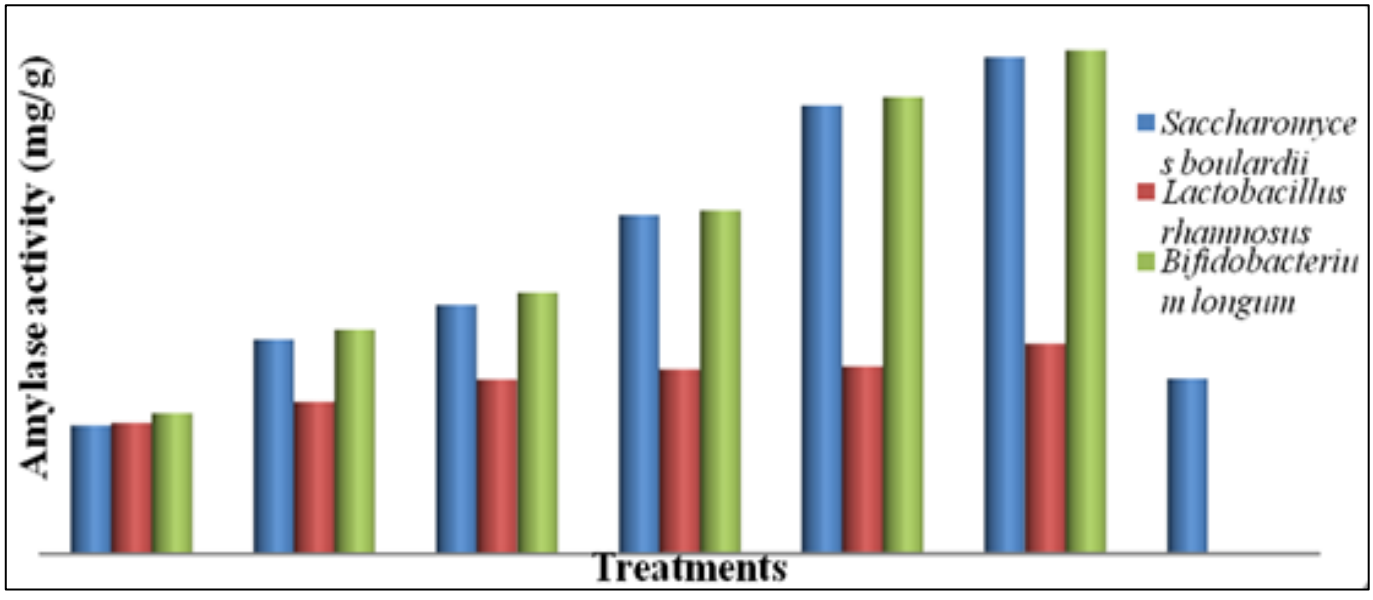

Fig 1: Effect of probiotics on amylase activity of double hybrid silkworm

Similarly, feed supplementation of 3% *Bifidobacterium longum* showed maximum invertase activity of 42.73 mg/g followed by *Saccharomyces boulardii* of 41.89 mg/g and *Lactobacillus rhamnosus* of 30.42 mg/g. The control showed minimum activity of 22.34 mg/g (Fig 2). In the same way, Esaivani *et al.* (2014) [2] made an attempt to study the impact of mulberry leaf fortification with probiotic microorganism *Saccharomyces cerevisiae* on the enzymatic profile and the quantitative economic parameters of silkworm, *Bombyx mori* and the results indicated that there was increase in amylase and invertase activity in the digestive juice of the probiotic treated worms than the control with enhanced immunity and quality silk production.

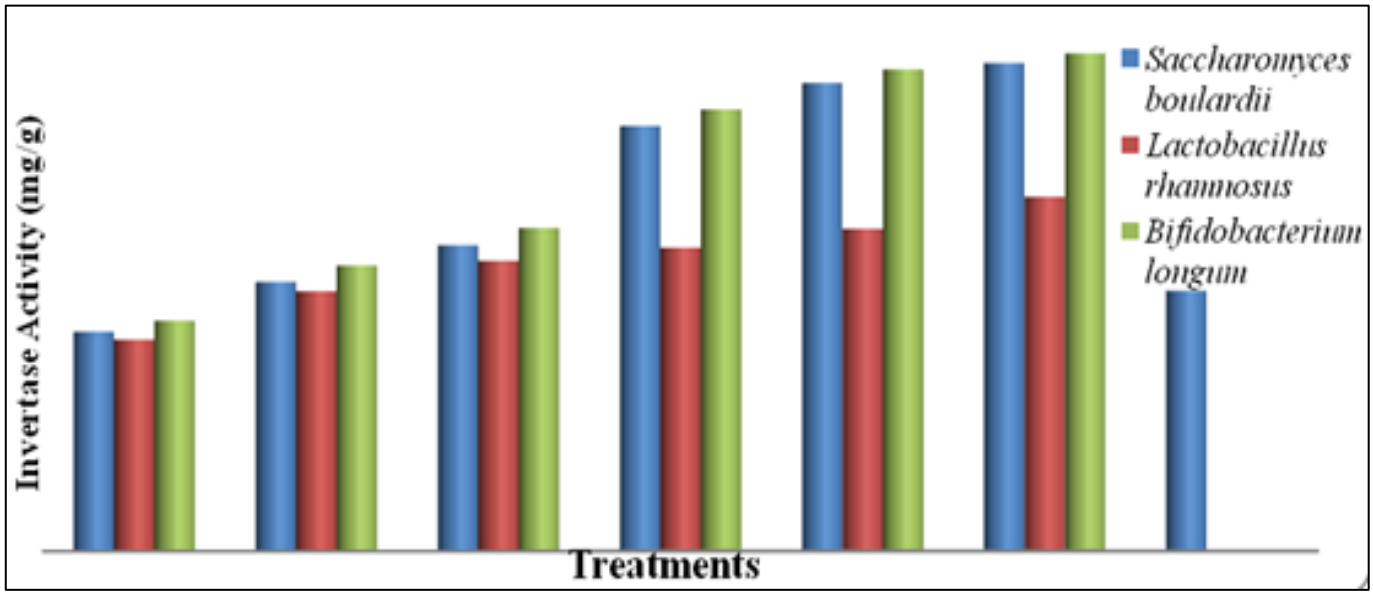

Fig 2: Effect of probiotics on invertase activity of double hybrid silkworm

Taha *et al*. (2017) [11] studied the enzymatic (Protease, Invertase and Amylase) activities of larvae feed on mulberry leaves supplemented with *Bifidobacterium bifidum* and *Saccharomyces cerevisiae*. Protease recorded highest activity upon the effect of *S. Cerevisiae* on hybrid 1 (70.77±3.65ug D,L-alanine/min) followed by *S. cerevisiae* on hybrid 2 (62.1±2.95*ug D,L-alanine/min/g)*. Highest invertase activity was recorded for hybrid 2 administrated with *S. Cerevisiae* (494.67±6.11ug glucose/min). And the highest amylase activity was recorded for hybrid 1 supplemented with *S. cerevisiae* (27.77±1.59 ug glucose/min/g). These studies showed that probiotics supplementation to silkworm larvae increased enzymatic activity which supports the present findings.

3.2. Impact of probiotics on silkworm economic traits

Supplementation of probiotics, *Saccharomyces boulardii*, *Lactobacillus rhamnosus* and *Bifidobacterium longum* to double hybrid silkworm increased economic characteristics. *Lactobacillus rhamnosus* @ 3% recorded highest cocoon weight (1.78 g), pupal weight (1.25 g), shell weight (0.29 g) and shell ratio (23.20%) when compared to control which recorded cocoon weight of 1.35 g, pupal weight of 1.07 g, shell weight of 0.20 g and shell ratio of 18.69% (Table 1).

Table 1: Impact of *Lactobacillus rhamnosus* on economic parameters of silkworm

Treatments	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell ratio (%)
0.5%	1.23^{e}	0.99^{d}	0.16^{de}	16.16^{f}
1.0%	1.30^{de}	1.01^{cd}	0.18^{d}	17.82^{e}
1.5%	1.39^{d}	1.05^{c}	0.19^{d}	18.09^{d}
2.0%	1.51^{c}	1.12^{bc}	0.21^{c}	19.26^{c}
2.5%	1.65^{b}	1.17^{b}	0.25^{b}	21.36^{b}
3.0%	1.78^{a}	1.25^{a}	0.29^{a}	23.20^{a}
Control	1.35^{d}	1.07^{c}	0.20^{c}	18.69^{cd}
SE(d)	0.0136	0.0128	0.0252	0.483
CD (0.05%)	0.0289	0.0302	0.0535	1.008

Effect of *Lactobacillus acidophilus*, on the growth of two strains of Thai silkworm, *Bombyx mori*, Nang Lai and Nang Lai X 108 were studied by Suraporn *et al.* (2015) [10] and *L. acidophilus* @ 10^{8} cells mL-1 was topically applied to mulberry leaves and fed to II, III, IV and V instars and observed quality parameters *viz.*, survival ratio, mature larval weight, pupation ratio, cocoon weight and cocoon shell ratio and the result confirmed that improvement of growth parameters was recorded in Nang Lai *vs* control, with a survival ratio of 92.66 ± 1.52% vs 84 ±1 .00%, a larval weight of 1.26 ± 0.05 g in the 5th instar (5th day) vs 1.18 ± 0.05 g, pupation ratio of 91 ± 1.00% vs 82.33 ± 1.52%, cocooning ratio of 91.33 ± 1.52% vs 85 ± 1.00%, cocoon weight of 1.08 ± 0.09 g vs 0.94±0.07 g, and cocoon shell ratio of 14.95 ± 0.06% vs 12.78 ± 0.15%.

The silkworm larvae fed on *Ricinus communis* leaves were treated with 0.5%,1%, 1.5%,2%,2.5%,3% concentrations of probiotic Darolac from 3rd instar onwards and its effect on food consumption, utilization and economic traits was studied to understand the efficiency of conversions and its potentials to increase the silk yield in eri silkworm. *R. communis* leaves treated with probiotics recorded various economic parameter like weight (7.96 gms/10 worms) pupal weight (4.06 gms), cocoon weight(4.61 g), shell weight (0.57 g), silk ratio (12.17%) and ERR (95%) showed increase in commercial qualities of cocoon at 2% concentration when compared with control (Sujatha *et al.*, 2015) [9]. Masthan *et al*. (2017) [5] conducted an experiment to determine the feed efficiency of silkworm fed mulberry leaves with four types of probiotics, Blue green algae, spirulina, Yeast, *Lactobacillus acidophilus*, *L. supergenes*. The results suggested that Blue green algae, Spirulina and yeast had better efficiency of food conversion when compared to other probiotics.

In the present study, *Saccharomyces boulardii* @ 3% recorded highest cocoon weight of 1.87 g, pupal weight of 1.46 g, shell weight of 0.43 g and shell ratio of 22.99% when compared to control which recorded cocoon weight (1.68 g), pupal weight (1.35 g), shell weight (0.35 g) and shell ratio (20.83%) (Table 2).

Table 2: Impact of *Saccharomyces boulardii* on economic parameters of silkworm

Treatments	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell ratio (%)
0.5%	1.51^{e}	1.22^{d}	0.29^{e}	19.20^{e}
1.0%	1.56^{d}	1.26^{cd}	0.31^{d}	19.87^{d}
1.5%	1.60^{d}	1.29^{c}	0.33^{cd}	20.62^{cd}
2.0%	1.68^{c}	1.32^{bc}	0.35^{c}	20.83^{c}
2.5%	1.79^{bc}	1.40^{b}	0.39^{b}	21.78^{b}
3.0%	1.87^{a}	1.46^{a}	0.43^{a}	22.99^{a}
Control	1.68^{c}	1.35^{bc}	0.35^{c}	20.83^{c}
SE(d)	0.07	0.05	0.11	1.00
CD (0.05%)	0.16	0.10	0.02	2.15

Mulberry leaves supplemented with commercial probiotics Flora-SB at the concentration of 1%, 3% and 5% were administered, starting with the first day, first feed of each instar. Probiotics with a concentration of 3% was very effective and recorded maximum food consumption (1.836 ± 0.07g), assimilation (1.544 ± 0.05 g), tissue growth (0.118 ± 0.002 g), RGR (54.06 ± 1.84%), cocoon weight (1.98 ± 0.09 g), shell weight (0.37 ± 0.01 g) and filament length (804.26 ± 23.12m) (Yeruva *et al*., 2020) [13]. These studies are in line with our present findings.

Bifidobacterium longum @ 3% recorded highest cocoon weight (1.88 g), pupal weight (1.25 g), shell weight (0.33 g) and shell ratio (26.40%) when compared to control which recorded cocoon weight of 1.46 g, pupal weight of 1.12 g, shell weight of 0.26 g and shell ratio of 23.21% (Table 3).

Table 3: Impact of *Bifidobacterium longum* on economic parameters of silkworm

Treatments	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)	Shell ratio (%)
0.5%	1.32 ^{d}	1.04^{e}	0.21^{d}	20.19^{e}
1.0%	1.37 ^{d}	1.07^{e}	0.23^{d}	21.49^{de}
1.5%	1.48^{c}	1.10^{d}	0.25^{cd}	22.72^{d}
2.0%	1.59^{b}	1.16^{c}	0.27^{c}	23.27^{c}
2.5%	1.79^{ab}	1.22^{b}	0.30^{b}	24.59^{b}
3.0%	1.88^{a}	1.25^{a}	0.33^{a}	26.40^{a}
Control	1.46^{c}	1.12^{cd}	0.26^{c}	23.21^{c}
SE(d)	0.01	0.02	0.482	0.502
CD (0.05%)	0.03	0.04	1.021	1.002

Shruti *et al.* (2019) [8] studied the impact of probiotics feed supplements *viz.*, *spirulina*, Azolla, yeast and soy milk at five different concentrations (1, 2, 3, 4 and 5%) on silkworm hybrid, PM × CSR-2. Among the probiotics tested, Azolla was found to be superior for effective rate of rearing. Cocoon weight on the day of 50 per cent spinning followed by soy milk and yeast in comparison with control.

Saranya *et al.* (2019) [7] investigated economic parameters of silkworm bivoltine double hybrid (CSR6 x CSR26) x (CSR2 x CSR27) fed on mulberry leaves fortified with *Staphylococcus gallinarum* strain SWGB 7 and *Staphylococcus arlettae* strain SWGB 16. *Staphylococcus gallinarum* Strain SWGB 7 (108 cfu/ml) recorded maximum larval weight (4.12 g), effective rate rearing (96.36%), cocoon weight (1.97 g), shell weight (0.37 g), pupal weight (1.60 g), shell ratio (18.78%), silk productivity (4.81 g), filament length (1170.84 m), filament weight (0.31 g) and finer denier (2.38) besides reduced larval mortality (3.64%) due to disease incidence compared to control.

Among three probiotics, *Bifidobacterium longum* recorded maximum enzymatic activity and highest cocoon traits followed by *Lactobacillus rhamnosus* and *Saccharomyces boulardii*. Hence, the present investigation indicated that, there was profound increase in both enzymatic profile and economic traits of silkworm due to probiotic treatment than control.

4. Conclusion

Application of feed supplementation of probiotics, *Saccharomyces boulardii*, *Lactobacillus rhamnosus* and *Bifidobacterium longum* to double hybrid silkworm showed positive impact on enzymatic profile and increased the commercial characteristics of cocoon. Among the different concentrations tested, 3 per cent recorded maximum amylase and invertase activity and showed highest cocoon weight, pupal weight, shell weight and shell ratio.

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