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Impact of Mulberry Leaf Enriched with Leucine on Dehydrogenase Activities and Economic Characters of *Bombyx mori* L.

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Abstract

The growth and development of silkworm significantly regulated by nutrition. Like any other heterotrophic insects, the silkworm, *Bombyx mori* L. obtain water and other chemical compounds from the diet which determine growth and silk production. An attempt has been made in the present investigation to record the effect of mulberry leaf fortified with leucine at varied concentrations viz., 0.5, 1.0 and 1.5% on succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) activity levels and economic parameters in bivoltine double hybrids namely $FC_1 \times FC_2$ and $FC_2 \times FC_1$. The results of the study inferred that, both the hybrids expressed maximum activities of succinate and lactate dehydrogenase at 1.5% supplementation over remaining concentrations as well as control batch. The highest enzyme activities were recorded in the fat body tissue during 5th instar 6th day followed by 3rd and 1st day larvae. Further, higher larval weight, cocoon weight, shell weight, shell percentage, filament weight, filament length and renditta were recorded at 1.5% supplementation as compared to control batch.

***Key words*:** Silkworm hybrids, Leucine, Supplementation, Enzymes, Commercial parameters

The domesticated silkworm, *Bombyx mori* L derive nutrients from mulberry leaf for the consolidation of biomass in the larval stage and utilize energy reserve during non-feeding stage of metamorphosis [1]. By and large, the quality and quantity of silk produced by this sericigenous insect mainly decided by mulberry leaf nutrients, environmental and edaphic factors, agronomical practices besides its genetic endowment [2-3]. The nourishment of silkworm with quality dietary nutrients is most important component of nutrition which promotes metabolic process inturn enhance larval growth and silk production [4]. Perhaps, utilization of dietary nutrients is proportional to synthesis of silk proteins in the silk gland [5]. Hence it is obvious that, exogenous supplementation of nutrients through the diet often recommended to enhance economic parameters of the silkworm by using various fortifying agents with mulberry leaf viz., honey and lemon, folic acid, ferrous magnesium sulphate, ecdysteroids, alfalfa [6-10]. The *B.mori* derived 72-86% of amino acid from the mulberry and nearly about 60% of the absorbed amino acids are utilized for synthesis of silk protein [11]. Moreover, the dietary proteins and amino acids not only regulate growth and silk production, but also digestibility and protein concentration in the haemolymph [12]. It is well document that *B. mori* require ten essential amino acids and are mostly obtained by diet [13]. A quite a good number of reports are available on exogenous supplementation of amino acids such as phenyl alanine, valine, methionine, glycine, alanine, glutamine and threonine by the diet at varied concentrations which enhance larval growth, economic parameters, enzyme activities and synthesis of biomolecules in the silkworm [14-18].

The Succinate dehydrogenase (SDH) found in mitochondria of different tissue of insects including *B. mori*. It establishes connecting link between citric acid cycle and electron transport chain oxidation and play a pivotal role in carbohydrate metabolism. On the other hand, lactate dehydrogenase (LDH) is a glycolitic enzyme responsible for reversible conversion of lactate to pyruvate with the aid of NAD found abundantly in the fat body tissue of insects. In *Bombyx mori* L, elevated levels of Succinate dehydrogenase (SDH) and LDH activities were noticed when larvae supplemented with tryptophan and riboflavin as compared to controls and their activities differ sharply among different silkworm breeds [19], [29]. Furthermore, Succinate dehydrogenase (SDH) expressed positive correlation with respect to commercial parameters of silkworm [20]. However, information on mulberry leaf fortified with leucine on dehydrogenase enzymes and economic parameters is meager. Hence, current investigation was undertaken to record changes in dehydrogenase activities as well as economic parameters during the supplementation of leucine with mulberry leaf.

MATERIALS AND METHODS

The bivoltine double hybrids namely $FC_1 \times FC_2$ and $FC_2 \times FC_1$ were selected for the present investigation and larvae were reared as per the method [21].

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Supplementation of leucine

The leucine at varied concentrations viz., 0.5, 1.0 and 1.5% were prepared with distilled water and sprayed on ventral surface of mulberry leaves and surface dried under shade before feeding to the silkworms. For the experimentation purpose, silkworm larvae divided into four batches viz., batch I (T_1), batch II (T_2) and batch III (T_3) were reared with mulberry leaf supplemented with leucine at 0.5, 1.0 and 1.5%, respectively. The control batch (T_4) was maintained parallelly and larvae were reared on mulberry leaves sprayed with distilled water. The treated leaves were fed to silkworms once in a day during IV and V instars. In each treatment, three replications were maintained each with 100 larvae along with control. The fat body tissue was collected by dissecting V instar 1st, 3rd and 5th day old larvae of respective treatments and control batch. The tissue homogenate of 1% was prepared with distilled water. After centrifugation the supernatant was collected and used for estimation of dehydrogenase enzymes. In addition, larval weight, cocoon weight, shell weight, shell percentage, filament length, filament weight, denier and renditta were also recorded. A minimum of twenty larvae were used from each treatment to measure succinate and lactate dehydrogenase activities.

Estimation of succinate and lactate dehydrogenase enzymes

The SDH and LDH activities were estimated as suggested by [22]. One ml of tissue extract was incubated with 1ml of sodium succinate, 1ml of phosphate buffer and 1ml of INT at 37 °C for 1 h. For LDH, lithium lactate was used as a substrate. The enzyme activities were stopped by adding 6ml of glacial acetic acid and 6ml of toluene. Finally, the test tubes were kept in refrigerator for overnight and OD was measured at 495nm using spectrophotometer against prepared blank. The standard graph was used for calculation and enzyme activities were expressed in terms of µ moles of formazan /g protein/h.

The commercial parameters such as shell ratio, filament length, denier and renditta were calculated by using following formulae:

$$\text{Shell ratio (\%)} = \frac{\text{Shell weight (g)}}{\text{Cocoon weight (g)}} \times 100$$

Filament Length (L) = R × 1.125

R = Number of revolutions recorded by an eprouvette
1.125 = Circumference of epprouvette in meter.

$$\text{Denier} = \frac{\text{Total weight of reeled silk (g)}}{\text{Total length of reeled silk (m)}} \times 9000$$

It denotes thickness of silk filament

$$\text{Renditta} = \frac{\text{Weight of cocoon reeled (g)}}{\text{Weight of raw silk (g)}}$$

Unit quantity of cocoons required to produced one unit of raw silk.
The obtained data were compiled and statistically analyze by standard methods.

RESULTS AND DISCUSSION

Influence of leucine supplementation on dehydrogenase enzymes

In the living organisms, various metabolic functions are carried out at cellular level by amino acids. The amino acids and proteins are the main sources of nitrogen which not only regulate growth but also silk production. The silkworm larvae utilize about 65 per cent of nitrogen during fifth instar for the production of silk [23-24]. It is the diet determine the quantity of free amino acids in the haemolymph. Moreover, increased free amino acid contents in larval haemolymph during supplementation of amino acids by the diet (mulberry leaf) because of synthesis of amino acids by non-protein sources such as glucose and fatty acids [25]. In *B. mori,* the function of fat body is analogue to vertebrate liver in which various metabolic process take place as haemolymph is close contact with fat body, silk gland and other tissues which helps to mobilize amino acids and other metabolites [26]. The overall growth of silkworm and synthesis of silk proteins are in fine tune between anabolic and catabolic processes. The leucine ($C_6H_{13}No_2$) is most potent ketogenic essential amino acid is cleaved catabolically by transmination, decarboxylation and dehydrogenation to form acetoacetate and acetyl-CoA [27]. Perhaps, synthesis of acetyle-CoA not only by metabolism of leucine but also by oxidation of fatty acids, amino acids and pyruvate from carbohydrates. The acetyle-CoA often regarded as a focal point in metabolism and is formed from coenzyme A (CoA). It is widely utilized at cellular level such as oxidation of glucose in the citric acid cycle, synthesis of cholesterol, fatty acids and steroid hormones [28]. The data pertaining to enzymes clearly depicts that the silkworm larvae $FC_2 \times FC_1$ reared on mulberry leaf fortified with leucine registered maximum activity levels of both SDH and LDH in fat body tissue at 1.5% concentration (5.45 µm of formazan/ g protein /h) and (5.31 µm of formazan / g protein /h) over respective controls (4.19 µm of formazan) and (4.17 µm of formazan) during 5th instar 5th day followed by 3rd and 1st day. Similar results were also observed in $FC_1 \times FC_2$ at 1.5% concentration (Fig 1-2).

The SDH belongs to class oxidoreductases which metabolize succinate into fumarate and during this process one hydrogen ion and two electrons are transfer to FAD. In TCA cycle, SDH transfer electron to cytochrome-b of respiratory chain and such association facilities quick electron transfer for oxidation of glucose or glycogen. Similarly, LDH is a glycolytic enzyme which converts lactic acid into pyruvate in which one hydrogen ion and 2 electrons are transfer to NAD. The significant variations were noticed with respect to SDH and LDH activities in insects including silkworm breeds and even at different stages of its life cycle during supplementation of nutrients [19]. It is clear from the current investigation is that both the silkworm hybrids expressed higher levels of SDH and LDH activities with higher concentration (1.5%) of leucine supplementation than lower concentrations (0.5 and 1.0%) as compared to control. The increase in SDH activity in the leucine supplemented batches might be due enhanced synthesis of acetyl-CoA which diffused into inner membrane of mitochondria to the sight of TCA cycle which in turn accelerate biological oxidation. Similarly, it is presumed that elevation in LDH activity in the larvae supplemented with leucine has resulted in increased synthesis of acetyl-CoA from the pyruvate. These results are in conformity with the observations of previous investigations [29] where in silkworm hybrids reared on mulberry leaves fortified with tryptophan at varied concentrations expressed increase in levels of SDH and LDH activities over control. Likewise, FC_1 and FC_2 silkworm larvae administrated with pyridoxine at different concentrations viz., 100, 500 and 1000ppm has resulted enhanced SDH activity when compared to control. However, the hybrid $FC_2 \times FC_1$ expressed maximum levels of SDH and LDH activities compared to $FC_1 \times FC_2$ as it has a greater potential to utilize the additional supplementation of leucine. These results are in agreement with earlier studies [29] who has reported the utilization of tryptophan varies among silkworm hybrids. Further, SDH and LDH activities gradually increased from 5th instar 1st day followed by 3rd and 5th day. It clearly depicts that mature larva require more amount leucine and hence progressive increase in enzyme activities were noticed. The elevated levels of SDH and LDH were observed as the larval age proceeds during 5th instar 1st day to 5th day during supplementation of tryptophan [38].

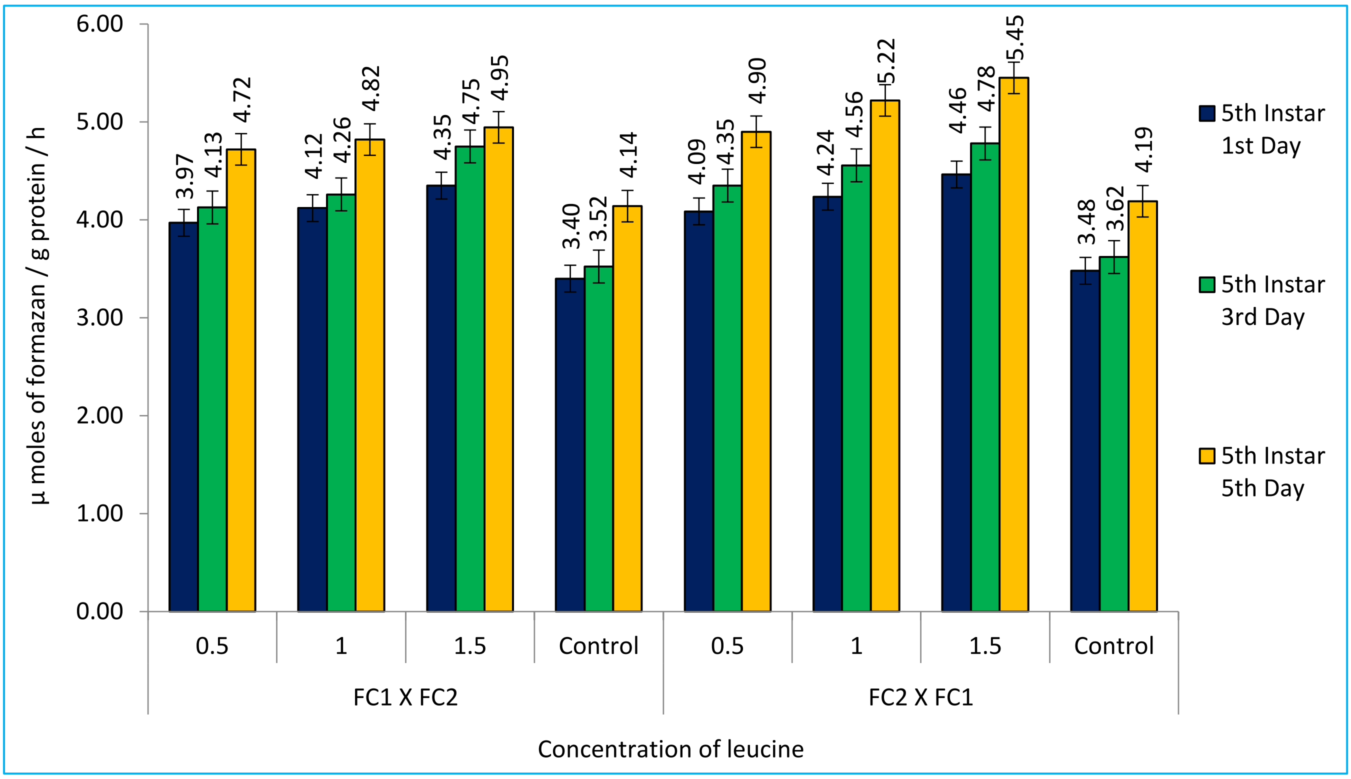

Fig 1 Effect of mulberry leaves supplemented with leucine at varied concentration on SDH activity in fat body of bivoltine double hybrids

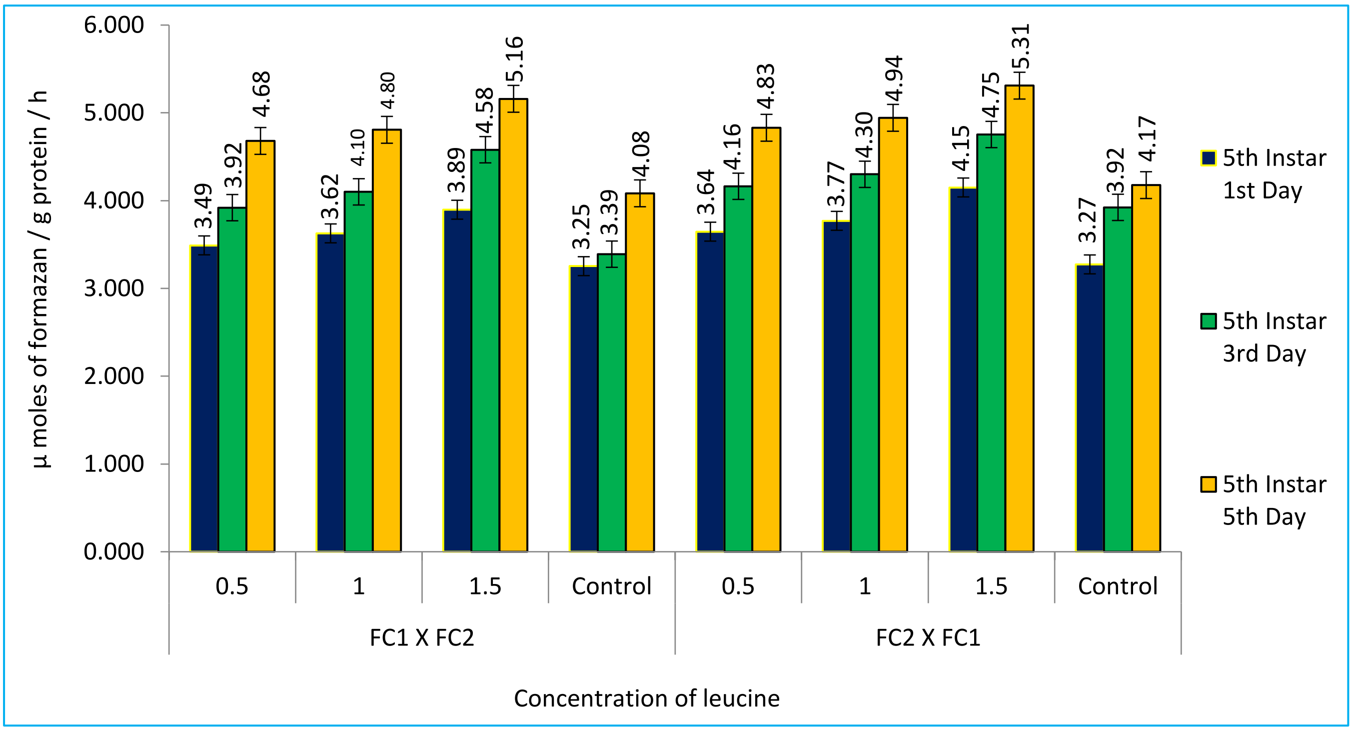

Fig 2 Effect of mulberry leaves supplemented with leucine at varied concentration on LDH activity in fat body of bivoltine double hybrids

Matured larval weight

Generally, proteins and amino acids supplements promotes growth and development which leads increased larval weight. Hence, gain in larval weight might be due to additional supplementation of leucine by the diet. Like other lepidopteran larvae, silkworm also exhibit intense feeding activity particularly during fifth instar and obviously it is one of the reasons in gaining larval weight [30]. Silkworm hybrids reared on mulberry leaves fortified with leucine at different concentrations exhibited notable influence on larval weight. The $FC_2 \times FC_1$ and $FC_1 \times FC_2$ expressed more larval weight of 4.85 and 4.72g (Table 1) when worms supplemented with leucine at 1.5% concentration against controls. The present findings are in harmony with the previous observations [31] who has reported that the silkworm hybrids supplemented with phenylalanine at 1.5% concentration exerted higher larval weight. Moreover, fat body and integument synthesis large amount of proteins from amino acids and their accumulation leads to gain in larval weight [32]. Similar results were observed which improves larval weight by the supplementation of amino acids such as methionine, alanine, arginine, tryptophan and glycine [33-37].

Table 1 Impact of mulberry leaves supplemented with leucine at varied concentrations of larval and cocoon characters

Hybrid	Concentration (%)	Larval weight (g)	Cocoon weight (g)	Shell weight (g)	Silk percentage (%)	Filament length (m)	Filament weight (g)	Denier	Renditta
$FC_1 \times FC_2$	0.5	4.24±0.02	2.05±0.01	0.33±0.03	16.09±0.05	985±2.08	0.32±0.01	2.92±0.09	6.41±0.09
	1	4.51±0.02	2.12±0.03	0.36±0.02	16.98±0.02	1050±2.00	0.34±0.01	2.91±0.08	6.23±0.08
	1.5	4.72±0.01	2.20±0.01	0.38±0.02	17.27±0.02	1150±5.00	0.36±0.01	2.81±0.03	6.15±0.04
	Control	3.95±0.05	1.85±0.03	0.29±0.01	15.67±0.04	880±5.00	0.27±0.01	3.10±0.01	6.82±0.08
$FC_2 \times FC_1$	0.5	4.55±0.05	2.14±0.02	0.36±0.01	16.82±0.05	1002±4.00	0.33±0.01	2.90±0.03	6.33±0.08
	1	4.62±0.02	2.26±0.02	0.40±0.01	17.69±0.02	1125±3.00	0.37±0.01	2.80±0.01	6.10±0.03
	1.5	4.85±0.05	2.35±0.03	0.42±0.01	17.87±0.05	1225±1.00	0.39±0.01	2.79±0.03	6.02±0.05
	Control	4.10±0.01	1.95±0.01	0.31±0.01	15.89±0.05	895±3.00	0.29±0.02	2.91±0.01	6.72±0.07

Cocoon weight

The cocoon weight is an important economic parameter differ among voltine groups. It depends upon ability of silkworm breeds which converts mulberry nutrients into cocoon formation. Supplementation of leucine at varied concentrations in silkworms registered encouraging results on cocoon weight. The larvae reared on leucine at 1.5% expressed higher cocoon weight of 2.35 and 2.20g in $FC_2 \times FC_1$ and $FC_1 \times FC_2$, respectively (Table 1). The increase in cocoon weight in both the breeds might be due to absorption of leucine by the body cells and get assimilated into cellular structure. These results are on par with the earlier findings [33] who have noticed that increased cocoon weight by the silkworm BSRI 83/3 reared on mulberry leaves extra foliated with methionine and tryptophan at 500ppm over remaining concentrations viz., 250, 750 and 1000ppm. This type of trend was also noticed with lycine, alanine, cysteine and glycine at 2% registered highest cocoon weight of 2.835, 2.695, 2.636 and 2.548g, respectively against control (2.254g) [37]. Likewise, worm nourished with alanine and asparagine at 0.2% exerted higher cocoon weight.

Shell weight

Shell weight is an important commercial character which denotes actual silk content of cocoon. It is evident from the data is that silkworms treated with leucine at 1.5% recorded higher shell weight (0.42g) in $FC_2 \times FC_1$ and (0.38g) $FC_1 \times FC_2$ over respective controls (0.31 and 0.29g) (Table 1). It is presumed that increase in shell weight is due to utilization of leucine which might have enhance synthesis of silk proteins in turn reflects on shell weight. These results corroborate the earlier studies [38] who have opined that silkworm larva MU11 and MU303 reared on mulberry leaves enriched with methionine at 0.5% concentration recorded higher shell weight of 0.217 and 0.215mg over controls (0.160 and 0.162 mg), respectively. Similarly higher shell weight was obtained with phenylalanine supplementation [39-40]. Furthermore, worms treated with threonine at the rate of 2% exerted higher shell weight and with proline and amino acids mixture [41-42].

Shell ratio

It indicates silk productivity of the silkworm races/breeds and helpful in fixing cocoon price. A concentration of leucine at 1.5% has resulted in higher shell ratio of 17.87 and 17.27% in $FC_2 \times FC_1$ and $FC_1 \times FC_2$, respectively (Table 1) when compared to control batches. The current observations are in conformity with the findings of previous investigations [14] where in silkworm hybrids $FC_1 \times FC_2$, FC_2 and FC_1 were nourished with mulberry leaf fortified with phenylalanine at 1.5% registered higher shell percentage over respective controls. Similarly, larva fed on mulberry leaves enriched with lycine, alanine, cysteine and glycine recorded higher shell weight at 2.5% and with tryptophan at 550ppm concentration [33], [37].

Filament length

It is one of the quantitative parameters varies among silkworm breeds [43]. The silkworms reared on mulberry leaf extra foliated with leucine at varied concentrations registered positive impact on filament length. Both hybrids FC_2 X FC_1 and FC_1 X FC_2 treated with leucine at 1.5% registered longer filament length of 1225 and 1150m over control batches (895 and 880m) (Table 1). These results are on par with earlier studies [31] who has concluded that bivoltine hybrids FC_2 and FC_1 treated with phenylalanine at 1.5% expressed longest filament length as against respective controls. Similar result was observed with lycine, alanine, cysteine and glycine [37] and with amino acid mixture (proline) at 0.01% [44].

Filament weight

This character is mainly decided by quantum of mulberry leaf fed by silkworm. The worm reared on mulberry leaves fortified with leucine recorded marked improvement with respect to filament weight as compared control. The maximum filament weight of 0.39 g ($FC_2 \times FC_1$) and 0.36 g ($FC_1 \times FC_2$) were obtained with leucine at 1.5% as against respective controls (0.29 and 0.27g) (Table 1). The present findings are in agreement with the previous observations [33] who have opined that larva treated with methionine and tryptophan scored higher filament weight. Similar trend was also noticed when worms are nourished with methionine at 0.5% and with phenylalanine, valine and methionine at 1.5% [14], [38].

Denier

It is one of the fascinating quality parameters which denotes thickness of the cocoon silk filament and varies along its length [45]. Generally, finer or lower denier silk is preferred in weaving sector. It is evident from current investigation is that silkworms treated with leucine at 1.5% expressed lower denier of 2.79 and 2.81 in $FC_2 \times FC_1$ and $FC_1 \times FC_2$ hybrids, respectively (Table 1). The present findings are comparable with those of earlier researchers [29], [33] who have observed that the larvae reared on mulberry leaves fortified with tryptophan at 0.5% expressed finer denier against control. This type of trend was also observed with amino acid mixture at 0.01% (2.42) over control (2.50) [44].

Renditta

This parameter denotes total available silk obtained from cocoons and lower value is preferred by the silk industry. The data pertaining to renditta registered encouraging results when silkworm larvae nourished on mulberry leaves enriched with leucine at different concentrations. The lowest renditta of 6.02 ($FC_2 \times FC_1$) and 6.15 ($FC_1 \times FC_2$) were obtained with leucine supplementation ment at 1.5% concentration (Table 1). The improvement for this parameter was noticed in the larvae treated with leucine. The current observations are in concurrence with the findings of previous investigations [29] who has obtained low renditta when the larvae treated with tryptophan at 1.5% concentration. Similarly, FC_1 and FC_2 larvae reared on mulberry leaf fortified with phenylalanine at 1.5% exerted lowest renditta over respective controls [31]. Likewise, administration of silkworm larvae with phenylalanine, methionine and valine at 1.5% registered lowest renditta [14]. Overall, these observations suggest that amino acid supplementation, particularly at a concentration of 1.5%, can be a viable strategy to enhance the efficiency of silk production by reducing renditta.

CONCLUSION

The results of current investigation inferred that; the silkworm larvae nourished with mulberry leaves supplemented with leucine at 1.5% enhance dehydrogenase enzyme activities as well as commercial parameters. Our findings could be utilized for the formulation of foliar spray and even for preparation of synthetic diets.

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