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SYNERGISTIC EFFECT OF HONEY AND LEMONJUICE-ENRICHED MULBERRY DIETS ON THE DIGESTIVE METABOLISM OF THE SILKWORM, *BOMBYX MORI*

Zoology

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ABSTRACT

Synergistic impact of honey and lemon juice-enriched mulberry diets has been studied on the digestive metabolism of fifth instar larval *Bombyx mori*. The study focused on the digestibility of proteins, carbohydrates, sucrose and cellulose by their digestive enzymes in the larval midgut wall and midgut lumen compartments and biomass accumulation in the gut wall. The honey and lemon juice-enriched diets showed positive impact on protease activity and protein digestion, but did not show discernable effect on the activity levels of α-amylase, sucrase and cellulase and the digestibility of carbohydrates, sucrose and cellulose. Nevertheless, they significantly reinforced the digestive mass accumulation in gut wall cells in accordance with the Hutchinson's investment principle. Further, as evidenced by higher growth rates in the digestive parameters during first five days of the fifth instar regime, the rate of biomass accumulation is fine-tuned by the timing of acquisition of critical larval body size determinants.

KEYWORDS

Bombyx mori, Carbohydrates, Cellulose, Digestive enzymes, Proteins, Sucrose

INTRODUCTION

The honey and lemon juice are used as exogenous dietary nutrients for the silkworm, with promising implications for larval growth, metabolism, silk production and economic traits of sericulture (Iglesias *et al.,* 2004; Sivaprasad and Thulasi, 2014; Thulasi and Sivaprasad, 2014). The honey is a natural sweetener and a repository of sugars (82%), proteins, enzymes, free amino acids, vitamins and many trace elements (Ball David, 2007). The lemon juice is a natural extract of *Citrus limen* (Family: Rutaceae) and the most popular multi-factorial nutrient that comprises ascorbic acid (Vitamin-C) in large quantities (64%) along with B-complex vitamins (13%), carbohydrates, sugars, lipids, proteins, minerals and some organic acids (Markus and Sass, 2003; Albertini *et al.*, 2006). The positive role of honey and lemon juice in stimulating the digestive function and biomass accumulation in silkworm has unveiled new vistas for further research in sericulture (Madhavi and Siva Prasad, 2022; Saritha and Siva Prasad, 2022).

In the mulberry sericulture industry, ***Bombyx mori*** is described as the biological machine that converts the dietary mulberry leaf to silk. Its gut wall and gut lumen compartments play pivotal role in processing the mulberry leaf and channelizing the dietary resources towards this end. In this process, the mulberry diet is subjected to chemical treatment in the larval midgut, before it is channelized for silk production (Cermenati *et al.*, 2007). While the glandular epithelium of the gut wall synthesizes and secrets digestive enzymes (proteases, amylases and lipases etc), the gut lumen receives the ingested food materials (proteins, lipids, carbohydrates, cellulose, pectin, vitamins, minerals and ions) and processes them through digestion and absorption (Terra and Ferreira, 2005; Xia *et al.,* 2007; Anand *et al*., 2010). As suggested by Ueda (1982), it is imperative to explore the possibility of improved digestive metabolism and biomass accumulation in the larval tissues of *B. mori* under the influence of quality nutritive diet for profitable outputs in sericulture. Therefore, the present investigation was taken up with a view to ascertain the synergistic effect of honey and lemon juice-enriched mulberry diets on digestive metabolism of silkworm, with particular reference to biomass accumulation during fifth instar larval development.

EXPERIMENTAL

Test Species & Experimental Design

The present investigation was carried out on the Fc_1 x Fc_2 hybrid silkworm, *Bombyx mori L* (Lepidoptera: Bombycidae). The experiment was designed to analyze the quantitative levels of total proteins, total carbohydrates, sucrose and cellulose together with the activity levels of their corresponding digestive enzymes such as protease, α-amylase, sucrase and cellulase under the impact of honey and lemon juice-enriched mulberry diets. The silkworms were reared in separate wooden trays on a standard diet (100%) of V_1 variety of mulberry leaves with 3 feeds per day at 6AM, 12 PM and 6PM, under standard environmental conditions of 28°C, 85% RH, 12h light and 12 h dark conditions. After the second molt, the third instar larvae were divided into four nutrient groups of 100 worms each and labelled them as zero dose control (ZDC), honey-fed experimental (HFE), lemon juice-fed experimental (LFE) and honey+lemon juice-fed experimental (HLFE) groups. While, the ZDC group was given normal mulberry diet, the HFE, LFE and HLFE groups were fed with mulberry leaves that were enriched either with honey (HFE) or lemon juice (LFE) or with both the nutrients (HLFE) at their 12.00 pm diet. The honey and lemon juice-enriched diets were prepared by soaking mulberry leaves, separately in 2% honey and 3% lemon juice in distilled water as per the standard minimum effective concentrations determined in our laboratory (Thulasi and Siva Prasad, 2014; Madhavi *et al*., 2018). The individual and synergistic effects of honey and lemon juice on the silkworm digestive metabolism was studied on the gut wall and gut lumen compartments simultaneously in the control (ZDC) and experimental (HFE, LFE & HLFE) groups. The gut wall tissue was isolated by mid-dorsal dissection of the silkworm larvae in ice cold Silkworm Ringer (Yamaoka *et al*., 1971). The gut content, which actually includes the digestive fluid was extracted through a hypodermic syringe by inserting it into the gut lumen. The digestive fluid, so collected was kept in a test tube under ice-cold conditions till the mulberry leaf pieces were settled at the bottom and then the supernatant was decanted and used for the biochemical assay.

Assay Of Digestive Biochemical Constituents

Total Proteins were estimated by the method of Lowry *et al.,* (1951) in 1% homogenates of gut wall tissue and 1:9 diluted digestive fluids in distilled water. The protease Activity was determined by the method of Davis and Smith (1955) in 5% homogenates of gut wall tissue and 1:19 diluted digestive fluid in ice cold distilled water. Total carbohydrates were estimated by the method of Carroll *et al.,* (1956) in 5% homogenates of gut wall tissue and 1:19 diluted digestive fluid in 10% trichloro acetic acid. The α-amylase activity was determined by the method of Bernfeld (1955) in 2% homogenates of gut wall and in 1:9 diluted digestive fluids in 0.05M acetate buffer. Sucrose levels were estimated by the method of Plumer (1978) in 5% homogenates of gut wall tissue and 1:19 diluted digestive fluid in ice cold distilled water. The sucrase activity was determined by the method of Ishaaya Swirski (1970) in 5% homogenates of gut wall tissue and 1:9 diluted digestive fluids in 0.05M ice cold phosphate buffer. Cellulose levels were estimated by the method of Updegroff (1969) in 5% homogenates of gut wall tissue and 1:9 diluted digestive fluids in acetic nitric reagent. The cellulase activity was determined by the method of Miller (1959) in 5% homogenates of gut wall tissue and 1:9 diluted digestive fluids in 0.05M phosphate buffer.

Statistical Analysis

The numerical data of digestive biochemical constituents were statistically analyzed by mean, standard deviation (SD), percent change and test of significance using M.S. Excel platform and online software packages (www, Graph pad. com / quick calcs / index cfm / and www.percent change com / index php). The instar-specific changes in their levels were expressed in terms of the overall growth

rates (OGRs). Further, with a view to assess day-to-day changes and arrive at meaningful conclusions, the levels of digestive substrates and the activity of digestive enzymes were also analyzed in terms of an innovative growth parameter called compound periodical growth rate (CPGR) as given by Siva Prasad (2012).

RESULTS AND DISCUSSION

The silkworm midgut consists of two compartments; the gut wall and gut lumen that help in digestion and absorption of the mulberry diet (Cermenati *et al*., 2007; Babu *et al*., 2009). The glandular epithelial cells of the gut wall synthesize and secrete digestive enzymes into the lumen compartment (Narayanaswamy and Shankar, 2010). The gut lumen is filled with the digestive fluid rich in biochemical constituents that emanate from the gut wall as well as the mulberry diet (Suryanarayana *et al*., 2002; Thirumalaisamy *et al*., 2009; Kalaivani *et al*., 2013). The digestive metabolism includes the digestion of the nutrients present in the mulberry diet and their final absorption and assimilation into gut wall tissues.

Nutrient diets enhance Protein levels and Protease Activity

In general, the levels of total proteins vis-a-vis protease activity recorded elevatory growth trends indicating increased protein digestion throughout the fifth instar regime and their levels were significantly boosted by the honey-and lemon juice-enriched mulberry diets all through.

Total Proteins

Gut Wall: In the gut wall tissue, the levels of total proteins increased from 3.27 mg/g to 13.46 mg/g, representing an OGR of abo~312% and a CPGR of 26.59% under normal dietary conditions. The nutrient-enriched mulberry diets caused elevations in total protein levels, both individually and synergistically. Accordingly, the protein profiles raised to 20.67 mg/g in HFE, 17.76 mg/g in LFE and 26.0 mg/g in HLFE. By doing so, the gut protein content recorded an OGR of ~532% and a CPGR of 35.98% in HFE, an OGR of ~443% and a CPGR of 32.58% in LFE and an OGR of ~695% and a CPGR of 34.43% in HLFE (Table 1).

Table 1: Synergetic effect of honey and lemon juice-enriched mulberry diet on total protein levels in the gut wall and gut lumen in *B. mori* during fifth instar development

Day	Statistical Tool	Total Proteins (mg/g in gut wall & mg/ml in gut lumen)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean S.D (±)	3.27 0.41**	0.80 0.10**	3.27 0.41**	0.80 0.10**	3.27 0.41**	0.80 0.10**	3.27 0.41**	0.80 0.10**
3	Mean S.D (±) PC (%)	7.39 2.8** 125.9	1.64 0.03** 104.9	13.61 0.5** 316.2	2.65 0.1** 231.2	11.95 0.33** 265.4	2.27 0.07** 183.8	19.34 0.35** 491.4	2.86 0.06** 257.5
5	Mean S.D (±) PC (%)	8.61 0.19** 16.5	1.89 0.07** 15.2	17.69 0.47** 29.9	2.76 0.04** 4.20	13.54 1.22** 13.30	2.41 0.01** 6.20	23.24 0.55** 20.2	3.29 0.04** 15.0
7	Mean S.D (±) PC (%)	13.46 0.96** 56.3	2.23 0.07** 17.9	20.67 0.38** 16.8	3.08 0.06** 11.6	17.76 0.46** 31.2	2.62 0.05** 8.70	26.0 1.01** 11.9	3.68 0.09** 11.9
Instar Mean		8.18	1.64	13.81	2.32	11.63	2.03	17.96	2.66
OGR (%)		311.6	178.8	532.1	285.0	443.1	227.5	695.1	359.9
CPGR %		26.59	18.63	35.98	25.19	32.58	21.86	34.43	6.40

** Statistically significant (P value <0.001); ** statistically not significant.*

Total protein values, expressed as mg / g wet weight of gut wall tissue or mg/ml of digestive fluid in gut lumen, is the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each period under ZDC (zero dose control), HFE (honey-fed experimental, LFE (lemon juice-fed experimental) and HLFE (honey=lemon juice-fed experimental) was calculated taking its previous value as the control. Both the overall growth rate (OGR) and compound periodical growth rate (CPGR) was separately calculated for the entire duration (7 days) of fifth instar, taking the initial ZDC value as the control.

Gut Lumen: Under normal dietary conditions, the gut lumen total protein levels increased from 0.80 mg/g to 2.23 mg/g and recorded an OGR of ~179% and a CPGR of 18.63%. Their levels further increased to higher levels under the impact of nutrient-rich diets. In HFE, the proteins raised to 3.08 and registered an OGR of ~285% and a CPGR of 25.19%. In LFE the levels rose to 2.62 mg/g and registered an OGR of ~228% and a CPGR of 21.86%. In HLFE, the protein levels scaled to the highest level of 3.68 mg/g, thus recording an OGR of ~360% and a CPGR of 6.40% during the 7-day fifth instar regime (Table 1).

Protease Activity

Gut Wall: The protease activity of the gut wall followed the increasing growth trends observed in its total protein levels (Table 2). In ZDC, its activity levels were enhanced from 0.36 µm/mg proteins/h to 0.66 µm /mg proteins /h and recorded an OGR of ~83% at the end of fifth instar. The activity levels of this enzyme were further boosted by all the three nutrient diets. Accordingly, in HFE, its levels increased to a higher level of 0.75 µm/ mg proteins/ h, recording an OGR of ~10% during the same period. At the same time, its activity peaked to a level of 0.72 µm and recorded an OGR of ~100% in LFE. Synergistically, the honey+lemon juice-enriched diet further boosted the enzyme activity to the highest level of 1.17 µm and thus recorded a higher OGR of 225% during the 7-day fifth instar larval regime. Surprisingly, the synergistic impact of honey and lemon juice on protease activity was uniquely significant as seen in HLFE, which recorded an exceptional elevation of ~79% on the last day of fifth instar (Table 2).

Table 2: Synergetic Effect Of Lemon Juice & Honey-enriched Mulberry Diets On Protease Activity In The Gut Wall And Gut Lumen In *B. Mori* During Fifth Instar Development

Day	Statistical Toll	Protease Activity (µ moles of tyrosine/ mg protein / hour)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean S.D (±)	0.36 0.01**	0.04 0.01**	0.36 0.01**	0.04 0.01**	0.36 0.01**	0.04 0.01**	0.36 0.01**	0.04 0.01**
3	Mean S.D (±) PC (%)	0.52 0.05** 44.4	0.05 0.02** 25.0	0.57 0.06** 58.3	0.04 0.01** 0.00	0.46 0.18** 27.8	0.05 0.01** 25.0	0.52 0.04** 44.4	0.05 0.01** 25.0
5	Mean S.D (±) PC (%)	0.55 0.03** 5.8	0.06 0.01** 19.9	0.62 0.03** 8.8	0.06 0.01** 49.9	0.66 0.04** 43.5	0.06 0.01** 20.0	0.65 0.02** 25.0	0.06 0.00 19.9
7	Mean PC (%) S.D (±)	0.66 0.08** 19.9	0.07 0.0** 16.7	0.75 0.02** 20.9	0.07 0.00** 16.7	0.72 0.03** 9.1	0.07 0.00** 19.9	1.17 0.01** 79.9	0.07 0.00** 16.7
Instar Mean		0.52	0.06	0.58	0.05	0.55	0.06	0.68	0.06
OGR (%)		83.3	75.0	108.3	75.0	100.0	50.0	225.0	75.0
CPGR %		10.63	9.78	13.01	9.78	12.25	9.78	21.71	9.78

** Statistically significant (P value <0.001); ** Statistically not significant. The protease activity was expressed as µ moles of tyrosine/ mg protein / hour. The remaining notations are the same as in Table 1.*

Gut Lumen: The protease activity in the digestive fluid of the mid-gut lumen is much lower than that in the gut wall cells. Nevertheless, it adopted similar elevatory path from the beginning to ending of fifth instar. In ZDC, its activity levels were enhanced from 0.04 µm/mg proteins/h to 0.07 µm /mg proteins /h and recorded an OGR of ~75% at the end of fifth instar. The nutrient diets have not altered the protease activity in the gut lumen. As such, it maintained at uniform OGR of ~75% all the experimental groups (Table 2).

Mean Protease Activity and Protein Digestion

Utilization of exogenous proteins is an important function involved in the growth and development of larvae and the production of quality silk. The gut wall cells represent the rich source of endogenous proteins involved in different functions. It is known that the gut wall cells synthesize and store several proteases including exopeptidases and dipeptidases during larval growth (Abudabos, 2012; Lokesh *et al*.,

2012). The gut content includes a variety of dietary proteins of mulberry leaf and a multitude of digestive enzymes such as proteases, carbohydrases and lipases emanating from gut wall cells and endosymbiotic microbes (Anand *et al*., 2010).

The proteases are weakly secreted into the gut lumen in the form of membrane bound vesicles covered by peritrophic membrane of the gut wall and hydrolyze high molecular weight dietary proteins into amino acids in the digestive fluid of the gut lumen, which are ultimately absorbed by the gut wall cells (Sarangi and Anitha, 2007). The compound periodical growth rates (CPGRs) depict the correct picture of day-to-day steady growth trends in protease activity vis-a-vis protein digestion. As shown in Tables 1 and 2, the instar-mean protein levels and the protease activity showed elevatory trends from day-1 to day-7 of the fifth instar, both in the gut wall and gut lumen. In ZDC, the gut wall protease activity reached a mean activity level of 0.52 µm of tyrosine /mg protein/h and registered a CPGR of 10.63% during the fifth instar regime. Their activity levels and CPGRs further improved under the impact of nutrient diets. As for instance, their activity levels scaled to 0.58 µm (CPGR: 13.01%) in HFE, 0.55 µm (CPGR: 12.25%) in LFE and 0.68 µm (CPGR: 21.71%) in HLFE. But surprisingly, the protease activity in the gut lumen remained static at 0.5 or 0.6 µm with a fixed CPGR of 9.78 in ZDC and nutrient (HFE, LFE, HLFE) groups (Table 2 & Fig. 1) The present growth trends in digestive parameters indicate that protein digestion might occur within the peritrophic membrane, while the membrane bound vesicles are still attached to the midgut wall. Thus, the gut wall protease activity indicates the prevalence of two types of digestions; intracellular and extracellular. Obviously, the former occurs within the glandular epithelium while the latter takes place in the digestive fluid of the gut lumen.

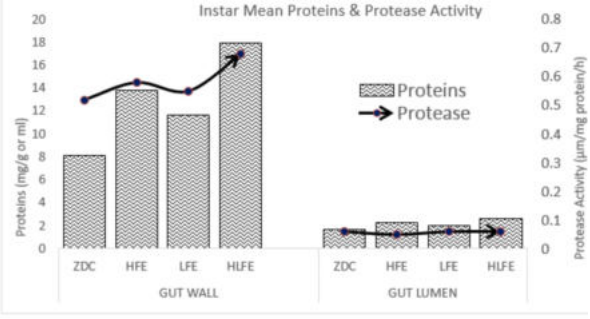

Fig. 1: *Synergistic effect of honey and lemon juice-enriched mulberry diets on mean protein leve and protease activity in the gut wall and gut lumen of B. mori during fifth instar larv development (Source: Tables 1 & 2)*

Nutrient Diets enhance Total Carbohydrate Levels, but do not alter Alpha-Amylase Activity

The levels of total carbohydrates and α-amylase activity showed opposing growth trends in the compartments of gut wall and gut lumen in both ZDC and three nutrient groups (Tables 3 & 4). While the levels of the former recorded elevatory growth trends, the latter showed declining trends throughout the fifth instar larval development.

Total Carbohydrates

Gut Wall: Within the gut wall tissue, the levels of total carbohydrates increased from 5.06 mg glucose/g to 12.52 mg glucose /g and recorded an OGR of ~147% and a CPGR of 16.30% in ZDC under normal dietary conditions. The nutrient mulberry diets, enriched with honey and lemon juice caused significant elevations in total carbohydrate levels. Accordingly, their levels were raised to 14.0 mg/g in HFE, 13.25 mg/g in LFE and 14.66 mg/g in HLFE. As such, the gut wall carbohydrate profiles recorded an OGR of ~177% and a CPGR of 18.48% in HFE, an OGR of ~162% and a CPGR of 17.40% in LFE and an OGR of ~190% and a CPGR of 19.40% in HLFE (Table 3).

Table 3: Synergetic Effect Of Lemon Juice & Honey-enriched Mulberry Diets On Total Carbohydrate Levels In The Gut Wall And Gut Lumen In *B. Mori* During Fifth Instar Development

Day	Statistical Toll	Total Carbohydrates (mg glucose /g or ml)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean	5.06	35.25	5.06	35.25	5.06	35.25	5.06	35.25
	S.D (±)	0.05*	1.36*	0.05*	1.36*	0.05*	1.36*	0.05*	1.36*
3	Mean	10.67	56.95	11.75	65.45	11.49	60.75	12.37	70.35
	S.D (±)	0.15*	1.06*	0.12*	0.66*	0.09*	0.9*	0.1*	1.42*
	PC (%)	110.9	61.6	132.2	85.7	127.1	72.3	144.5	99.6
5	Mean	11.21	60.60	13.13	75.45	12.48	67.45	14.06	78.6
	S.D (±)	0.11*	2.38*	0.3*	0.72*	0.1*	0.77*	0.08*	0.67*
	PC (%)	5.1	6.4	11.7	15.3	8.6	11.03	13.7	11.7
7	Mean	12.52	67.85	14.0	78.83	13.25	74.05	14.66	81.45
	S.D (±)	0.28*	1.86*	0.09*	1.37*	0.33*	0.44*	0.15*	0.82*
	PC (%)	11.7	11.9	6.6	4.5	6.2	9.8	4.3	3.6
Instar Mean		9.87	55.16	10.99	63.75	10.57	59.38	11.54	66.41
OGR (%)		147.4	92.5	176.7	123.6	161.9	110.1	189.7	131.1
CPGR %		16.30	11.53	18.48	14.36	17.40	13.17	19.40	14.98

** Statistically significant (P value <0.001); ** Statistically not significant.*

The values of total carbohydrates, expressed as mg / g wet weight of gut wall tissue or mg/ml of digestive fluid in gut lumen. The remaining notations are the same as in Table 1.

Gut Lumen: In the digestive fluid of gut lumen, the total carbohydrate levels were 5 to 6 times higher than those in the gut wall tissue. In ZDC, their levels increased from 35.25 mg glucose/ ml of digestive fluid to 67.85 mg glucose /ml, representing an OGR of ~93% and a CPGR of 11.53% under normal dietary conditions. Their levels were further elevated by the nutrient-rich diets. In HFE, they were raised to 78.83 mg/ml and registered an OGR of ~123% and a CPGR of 14.36%. In LFE the levels rose to 74.05 mg/ml and registered an OGR of ~110% and a CPGR of 13.17%. In HLFE, their levels peaked to 81.45 mg/ml, recording an OGR of ~131% and a CPGR of 14.98% in this raising phase (Table 3).

Alpha-Amylase Activity

Gut Wall: The α-amylase activity of the gut wall followed a declining growth trend during fifth instar (Table 4). In ZDC, its activity levels dropped from 5.23 µm of maltose/mg carbohydrates/h to 4.34 µm, recording a negative OGR of ~17%. Though the nutrient diets have slightly boosted the enzyme activity in some cases, its declining trend remained unaffected all through. Accordingly, in HFE, its levels fell to a lower level of 4.32 µm/h, with a negative OGR of ~17% during the same period. In LFE, its activity levels dropped to 4.08 µm/h, with a negative OGR of ~22% and in HLFE, its levels slumped to 4.27µm/h, with a negative OGR of ~1.84% during the 7-day fifth instar larval regime (Table 4). Further, the rate of decline in enzyme activity was more pronounced during the first half of fifth instar as evidenced by higher declining rates in its activity on day-3 in ZDC (~12%), HFE (~6.5%), LFE (~11%) and HLFE (~8%).

Table 4: Synergetic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On A-amylase Activity In The Gut Wall And Gut Lumen In *B. Mori* During Fifth Instar Development

Day	Statistical tool	α-Amylase Activity (µ moles of maltose /mg carbohydrate / hr)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean	5.23	0.09	5.23	0.09	5.23	0.09	5.23	0.09
	S.D (±)	0.10**	0.01**	0.10**	0.01**	0.10**	0.01**	0.10**	0.01**
3	Mean	4.58	0.08	4.89	0.08	4.66	0.07	4.79	0.07
	S.D (±)	0.20*	0.00**	0.16**	0.00**	0.22*	0.01**	0.08*	0.00**
	PC (%)	-12.4	-11.1	-6.5	-11.1	-10.9	-22.2	-8.4	22.2
5	Mean	4.56	0.08	4.64	0.07	4.61	0.07	4.49	0.07
	S.D (±)	0.26*	0.00**	0.12*	0.00**	0.11**	0.00**	0.08*	0.00**
	PC (%)	-0.4	0.00	-5.1	-12.5	-1.1	0.0	-6.3	0.00
7	Mean	4.34	0.08	4.32	0.07	4.08	0.06	4.27	0.06
	S.D (±)	0.13*	0.00**	0.10**	0.00**	0.11**	0.00**	0.09*8	0.00**
	PC (%)	-4.8	0.00	-6.9	0.00	-11.5	-14.3	-4.9	-14.3
Instar Mean		4.68	0.083	4.77	0.077	4.65	0.073	4.70	0.073

OGR (%)	-17.0	-11.1	-17.4	-22.2	-21.9	-33.3	-18.4	-33.3
CPGR %	-3.06	-1.94	-3.14	-4.10	-4.05	-6.53	-3.32	-6.53

** Statistically significant (P value <0.001); ** Statistically not significant.* α-Amylase Activity is expressed as *μ moles of maltose /mg carbohydrate h. The remaining notations are the same as in Table 1.*

Gut Lumen: The α-amylase activity of the gut lumen displayed similar declining growth trends during fifth instar (Table 4). In ZDC, its activity levels declined from 0.09 μm of maltose/mg carbohydrates/h to 0.08 μm and thus, recording negative OGR of ~11% during the instar regime. The nutrient diets have not significantly altered the enzyme activity. Accordingly, in HFE, its levels fell to a lower level of 0.07 μm/h and recorded a negative OGR of ~22%. In both LFE and HLFE, its activity levels dropped uniformly to 0.06 μm/h (OGR: - 33%) during the same period (Table 4). Further, the dropping rates of the enzyme activity was more pronounced during the first half of fifth instar as evidenced by larger negative growth rates in its activity on day-3 in ZDC (~11%), HFE (~11%), LFE and HLFE (~22% each).

Mean α-Amylase Activity and Carbohydrate Digestion

Alpha- amylase is one of the most important digestive enzymes, majorly synthesized and secreted by midgut cells and moderately synthesized by fore and hind gut cells during fourth and fifth instar larval stages under the stimulant effect of the starch present in the gut lumen (Terra and Ferreira, 2005; Zibaee *et al*., 2008). The starch is the chief carbohydrate moiety of the mulberry leaf, comprising repeated units of D-glucose that are connected by α-1, 4 glycosidic linkages that are cleaved by specific enzymes known as α-amylases that are synthesized and secreted by the midgut cells at regular intervals (Anand *et al*., 2010; Kaur *et al*., 2014). The α- amylase showed different activities with different rates of carbohydrate digestion in the gut wall and gut lumen. In general, its activity showed inverse relationship with increasing levels of total carbohydrates in both the compartment of the midgut. While, the mean carbohydrate levels increased, the corresponding α- amylase activities declined, indicating the slow process of carbohydrate digestion from day-1 to day-7 of the fifth instar (Tables 4 & Fig. 2). In ZDC, the gut wall α- amylase reached a mean activity level of 4.68 μ moles of maltose /mg carbohydrate/h, registering a negative CPGR of 3.06% during the fifth instar regime. Under the impact of nutrient diets, the gut wall enzyme activity levels projected either slight elevations or declines. In HFE, the enzyme activity climbed to 4.77 μ moles (CPGR: -3.14%), but declined to 4.65 μ moles (CPGR: -4.05%) in LFE. Once again its activity rose to 4.70 μ moles (CPGR: -3.32%) in HLFE. But surprisingly, in the gut lumen the mean α- amylase activity level, which stood at 0.083 μ moles (CPGR: -1.94%), slightly declined to 0.077 μ moles (CPGR: -4.10%) in HFE, 0.073 μ moles (CPGR: -6.53%), both in LFE and HLFE (Tables3, 4 & Fig. 2).

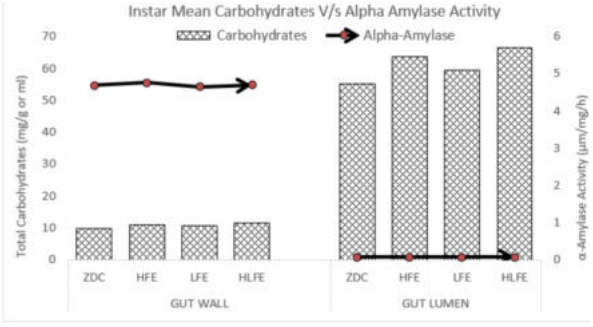

***Fig.2:** Synergistic effect of honey and lemon juice-enriched mulberry diets on total carbohydrate and α-amylase activity in the gut wall and gut lumen of B. mori during fifth instar larva development (Source: Tables 3 & 4)*

Nutrient Diets enhance Sucrose levels, but do not alter Sucrase Activity

The levels of sucrose and sucrase activity showed opposing growth trends in the compartments of gut wall and gut lumen in both ZDC and three nutrient groups (Tables 4 & 5). While the former recorded elevatory growth trends, the latter showed declining trends throughout the fifth instar.

Sucrose

Gut Wall: In the gut wall tissue, the sucrose levels increased from 6.84 mg glucose/g to 9.85 mg glucose /g and recorded an OGR of ~44% and a CPGR of 6.27% in ZDC, under normal dietary conditions. The nutrient-enriched mulberry diets caused significant elevations in sucrose levels throughout the fifth instar. The levels of this disaccharide raised to 13.26 mg/g in HFE, 11.32 mg/g in LFE and 14.62 mg/g in HLFE. As such, the gut wall sucrose levels recorded an OGR of ~94% and a CPGR of 11.66% in HFE, an OGR of ~66% and a CPGR of 8.76% in LFE and an OGR of ~114% and a CPGR of 13.50% in HLFE (Table 5).

Table 5: Synergetic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On Sucrose Levels In The Gut Wall And Gut Lumen In *B. Mori* During Fifth Instar Development

Day	Statistical Toll	Sucrose (mg glucose /g or ml)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean S.D (±) PC (%)	6.84 0.2* 	7.08 1.87* 	6.84 0.2* 	7.08 1.87* 	6.84 0.2* 	7.08 1.87* 	6.84 0.2* 	7.08 1.87*
3	Mean S.D (±) PC (%)	7.32 0.13* 7.0	7.75 0.79* * 9.5	8.62 0.19* * 26.0	14.73 0.67* 108.1	7.90 0.05* * 15.5	12.85 0.89* 81.5	9.67 0.14* * 41.4	18.15 0.79* 156.4
5	Mean S.D (±) PC (%)	8.60 0.13* 17.5	17.98 0.77* 132.0	10.47 0.22* 21.5	22.98 0.97* 56.0	9.32 0.16* 17.9	20.20 0.81* 57.2	11.51 0.15* 19.0	26.28 0.96* 44.8
7	Mean S.D (±) PC (%)	9.85 0.25* 14.5	22.93 0.35* 27.5	13.26 0.3* 26.6	28.80 0.39* 25.3	11.32 0.42* 21.5	26.48 0.88* 31.1	14.62 0.22* 27.0	31.38 0.4* 19.4
Instar Mean		8.15	13.94	9.80	18.40	8.85	16.65	10.66	20.72
OGR (%)		44.0	224.0	93.9	306.8	65.5	274.0	113.7	343.2
CPGR %		6.27	21.64	11.66	26.35	8.76	24.59	13.50	28.17

** Statistically significant (P value <0.001); ** Statistically not Significant. Sucrose values, expressed as mg of glucose/g wet weight of gut wall tissue or ml of digestive fluid in gut lumen. The remaining notations are the same as in Table 1.*

Gut Lumen: Most of the sucrose present in the digestive fluid of gut lumen has its origin from the mulberry leaf. In silkworm, the mid gut lumen maintains relatively higher levels of sucrose, compared to those in the gut wall cells. In the current study, its levels increased from 7.08 mg glucose/ ml of digestive fluid to 22.93 mg glucose /ml, representing an OGR of ~224% and a CPGR of 21.64% in ZDC. Their levels were further elevated by the nutrient-rich diets. In HFE, they were raised to 28.80 mg/ml and registered an OGR of ~307% at a steady CPGR of 26.35%. In LFE its levels rose to 26.48 mg/ml and registered an OGR of ~274% at a steady CPGR of 24.59%. In HLFE, the lumen sucrose levels peaked to the highest level of 31.38 mg/ml, thus recording an OGR of ~343% and a CPGR of 28.17% during the 7-day fifth instar regime (Table 5).

Sucrase Activity

Gut Wall: The sucrase activity of the gut wall followed a declining growth trend throughout the fifth instar. In ZDC, its activity levels declined from 7.64 μm of glucose/mg trehalose /h to 2.54 μm and recorded a negative OGR of ~67% and a negative CPGR of 16.77% at the end of fifth instar. The nutrient diets have retarded the final instar-end enzyme activity, while causing some minor day-to-day fluctuations.

As such the final activity levels of the enzyme stood at 2.01 μm/h in HFE, 2.11 μm/h in LFE and 1.74 μm/h in HLFE and this is associated with negative OGRs. The HFE recorded an OGR of -74%, the LFE an OGR of -72% and the HLFE, an OGR of -77% during the 7-day fifth instar larval regime. Further, the rate of decline in enzyme activity was more pronounced during the first half of fifth instar as evidenced by greater declining rates in its activity on day-3 and day-4 (-44% & -22%) in ZDC, (-41% & -52% in HFE, (-39% & -46%) in LFE and -47% and -34% in HLFE (Table 6).

Table 6: Synergetic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On Sucrase Activity In The Gut Wall And Gut Lumen In *B. Mori* During Fifth Instar Development

Day	Statistical tool	Sucrase Activity (µ moles of glucose /mg trehalose/ h)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean S.D (±)	7.64 0.21**	0.05 0.01**	7.64 0.21**	0.05 0.01**	7.64 0.21**	0.05 0.01**	7.64 0.21**	0.05 0.01**
3	Mean S.D (±) PC (%)	4.28 0.08** -43.9	0.04 0.01** -20.0	4.53 0.06** -40.7	0.05 0.01** 0.00	4.63 0.02** -39.4	0.05 0.01** 25.0	4.02 0.03** -47.4	0.05 0.00** 25.0
5	Mean S.D (±) PC (%)	3.33 0.06** -22.2	0.04 0.00** 0.00	2.17 0.07** -52.1	0.05 0.00** 0.00	2.50 0.07** -46.0	0.05 0.0** 0.00	2.64 0.08** 34.2	0.05 0.00** 0.00
7	Mean S.D (±) PC (%)	2.54 0.08** -23.7	0.04 0.00** 0.00	2.01 0.04** -7.4	0.05 0.00** 0.00	2.11 0.05** -15.6	0.05 0.00** 0.00	1.74 0.03** -34.1	0.05 0.01** 0.00
Instar Mean		4.45	0.043	4.09	0.05	4.22	0.05	4.01	0.05
OGR (%)		-66.8	-20.0	-73.7	0.00	-72.4	0.0	-77.2	0.0
CPGR %		-16.77	-3.65	-19.95	0.0	-19.30	0.0	-21.85	0.0

** Statistically significant (P value <0.001); ** Statistically not significant. Sucrase activity, expressed as µ moles of glucose /mg trehalose/ h. The remaining notations are the same as in Table 1.*

Gut Lumen: In the gut lumen, the sucrase activity either remained unchanged or slightly altered, both under normal and nutritionally modified conditions. By and large the enzyme activity levels stood at 0.04 or 0.05 µm of glucose/mg trehalose /h all through, a fact that reflects the maintenance of status-quo with reference to digestion of sucrose in the midgut of silkworm and it is not perturbed by external or internal factors (Table 6).

Mean Sucrase Activity and Sucrose Digestion

The sucrase is a carbohydrase that hydrolyses sucrose to glucose and fructose and it is widely distributed in the different regions of the digestive system (Kanekatsu, 1978). Among many other nutritive materials, the midgut synthesizes, stores, secrets and absorbs sucrose, which enhances the rate of feeding in *Bombyx mori* (Sumida *et al*., 1990). The glucose moieties so formed, are absorbed and converted to storage carbohydrates called glycogen and trehalose (Thompson, 2003).

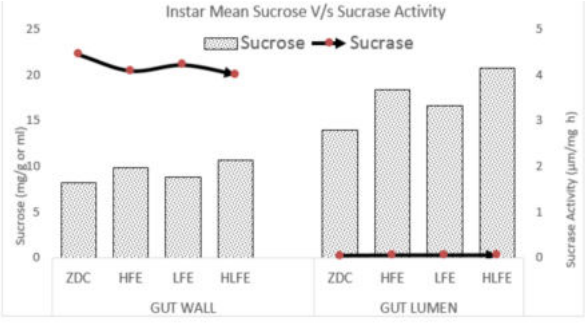

***Fig.3:** Synergistic effect of honey and lemon juice-enriched mulberry diets on sucrose levels and* **sucrase activity** *in the gut wall and gut lumen of B. mori during fifth instar larval development (Source: Tables 5 & 6)*

The sucrase presented differential activity in the two compartments of the midgut. In general, its activity showed inverse relationship with increasing levels of sucrose in gut wall as well as gut lumen. While, the mean sucrose levels increased, the corresponding sucrase activity levels declined, indicating the slow process of sucrose digestion from day-1 to day-7 of the fifth instar (Tables 5, 6 & Fig. 3). Under normal dietary conditions, the gut wall sucrase recorded a mean activity of 4.45 µ moles of glucose /mg trehalose/h and registered a negative CPGR of 16.77% during the fifth instar regime. Its activity further declined under the impact of nutrient diets and its decline was closely associated with negative CPGRs. The sucrase activity was brought down to 4.09 µ moles (CPGR: -19.95%) in HFE, to 4.22 µ moles (CPGR: -19.30%) in LFE and to a further low of 4.01 µ moles (CPGR: -21.85%) in HLFE. However, in the gut lumen the mean sucrase activity maintained a stable value of approximately 0.05 µ moles in ZDC, HFE, LFE and HLFE groups uniformly with a zero CPGR all through (Table 6).

Nutrient Diets Enhance Cellulose Levels, But Do Not Alter Cellulase Activity

The plant sugar, cellulose and its hydrolyzing enzyme projected contradictory growth trends during fifth instar. While the substrate levels increased considerably, the enzyme activity slumped sharply by the instar-end (Tables 7 & 8).

Table 7: Synergetic effect of honey and lemon juice-enriched mulberry diets on cellulose levels in the gut wall and gut lumen in *B. mori* during fifth instar development

Day	Statistical Toll	Cellulose (mg glucose /g or ml)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean S.D (±)	9.50 0.18**	5.36 0.07**	9.50 0.18**	5.36 0.07**	9.50 0.18**	5.36 0.07**	9.50 0.18**	5.36 0.07**
3	Mean S.D (±) PC (%)	10.23 0.25** 7.7	5.82 0.03** 8.6	11.59 0.12** 21.9	6.57 0.12** 22.6	11.25 0.11** 18.4	6.17 0.07** 15.1	12.72 0.37** 33.9	7.00 0.05** 30.6
5	Mean S.D (±) PC (%)	10.80 0.27** 5.60	6.38 0.04** 9.60	12.37 0.21** 6.70	7.14 0.04** 8.70	11.51 0.19** 2.30	6.81 0.04** 10.4	13.62 0.27** 7.1	7.50 0.11** 7.1
7	Mean S.D (±) PC (%)	11.64 0.12** 7.8	6.77 0.17** 6.1	14.08 0.32** 13.8	7.83 0.12** 9.7	12.66 0.37** 9.9	7.59 0.13** 11.5	15.58 0.22** 14.4	8.44 0.11** 12.5
Instar Mean		10.54	6.08	11.89	6.73	11.23	6.48	12.86	7.08
OGR (%)		22.5	26.3	48.2	46.1	33.3	41.6	64.0	57.5
CPGR %		3.44	3.97	6.78	6.52	4.90	5.97	8.59	7.86

** Statistically significant (P value <0.001); ** Statistically not significant. Sucrose values, expressed as mg glucose/g wet weight of gut wall tissue or ml of digestive fluid in gut lumen. The remaining notations are the same as in Table 1.*

Cellulose

Gut Wall: In ZDC, from an initial value of 9.50 mg glucose /g the gut wall cellulose levels rose to 11.4 mg glucose /g and registered an OGR of ~22% and a CPGR of 3.44% in its growth. The honey and lemon juice-enriched diets caused significant elevations in its levels. In HFE, the gut wall cellulose recorded a higher value of 14.08 mg/g and recorded an OGR of ~48% and a CPGR of 6.78% in its growth. At the same time its levels rose to ~13 mg/g and registered an OGR of ~33% and a CPGR of 4.90% in LFE. In HLFE, its levels rose to 15.58 mg/g and registered an OGR of ~64% and a CPGR of 8.59% on the last day of fifth instar (Table 7).

Gut Lumen: The cellulose levels of gut lumen are controlled by dietary uptakes. In ZDC, the rate of uptake of dietary cellulose into the gut lumen increased from 5.36 mg/g to 6.77 mg/g, which registered an OGR of ~26% and a CPGR of 3.97% by the end of fifth instar. During the same period, the nutrient diets caused considerable elevations in its levels. In HFE, the lumen cellulose levels recorded a higher value of 7.83 mg/g and recorded an OGR of ~46% and a CPGR of 6.52%. At the same time in LFE, its levels rose to ~8 mg/g and registered an OGR of ~42% and a CPGR of 5.970%. In HLFE, its levels touched the mark of 8.44 mg/g with an OGR of ~57% and a CPGR of 7.86% in its growth by the end of fifth instar (Table 7).

Cellulase Activity

Gut Wall: In ZDC, the cellulase activity declined from 7.69 µ moles of glucose /mg cellulose/ h to 2.54 µ moles of glucose /mg cellulose/ h, with negative growth rates (OGR: -67% & CPGR: -16.86%) during fifth instar. Under the impact of nutrient diets its negative growth trends remained unaffected. In HFE, the enzyme activity slumped to

2.10 µm and registered an OGR of -73% and a CPGR of 19.45% while in LFE, it dropped to 2.24 µm with an OGR of -71% and a CPGR of -18.58% in its growth. In HLFE, its activity level further dropped to 1.77 µm, representing an OGR of -77% and a CPGR of 21.72%. Significantly on day-2 of fifth instar, the cellulase recorded a great slump in its activity, with negative growth rates in ZDC (-45%), HFE (-65%), LFE (-66%) and HLFE (-61%) groups (Table 8).

Gut Lumen: In the gut lumen compartment, the cellulase activity declined from 0.18 µ moles of glucose /mg cellulose/ h to 0.09 µ moles of glucose /mg cellulose/ h, representing a negative OGR of ~-50% and a CPGR of -10.91% in ZDC. Under the impact of nutrient diets, the enzyme activity vis-à-vis its negative growth trends continued in all cases. In both HFE and LFE, the enzyme activity slumped to 0.08 µm and registered an OGR of -56% and a CPGR of -12.64% and in HLFE, it further dropped to 0.07 µm, representing an OGR of -61% and a CPGR of 14.56%. Great slump in enzyme activity occurred on day-2 of fifth instar, on which it was slashed by 50% in ZDC and LFE and 56% in HFE and HLFE (Table 8).

Table 8: Synergetic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On Cellulase Activity In The Gut Wall And Gut Lumen In *B. Mori* During Fifth Instar Development

Day	Statistical Toll	Cellulase Activity (µ moles of glucose /mg cellulose/ h)							
		ZDC		HFE		LFE		HLFE	
		Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen	Gut wall	Gut lumen
1	Mean S.D (±) 	7.69 0.20*	0.18 0.01*	7.69 0.20*	0.18 0.01*	7.69 0.20*	0.18 0.01*	7.69 0.20*	0.18 0.01*
3	Mean S.D (±) PC (%)	4.36 0.06* -44.6	0.09 0.00* -50.0	2.70 0.07* -64.9	0.08 0.00* -55.6	2.62 0.05* -65.9	0.09 0.01* -50.0	2.98 0.04* -61.2	0.08 0.01* -55.6
5	Mean S.D (±) PC (%)	3.29 0.05* -24.5	0.09 0.00* 0.00	2.29 0.03* -15.2	0.08 0.01* 0.00	2.60 0.04* -0.76	0.09 0.01* 0.00	2.16 0.05* -27.5	0.08 0.00* 0.00
7	Mean S.D (±) PC (%)	2.54 0.04* -22.8	0.09 0.00* 0.00	2.10 0.03* -8.3	0.08 0.00* 0.00	2.24 0.04* -13.8	0.08 0.00* -44.1	1.77 0.04* -18.1	0.07 0.00* -12.5
Instar Mean		4.47	0.11	3.69	0.11	3.79	0.11	3.65	0.10
OGR (%)		-66.9	-50.0	-72.7	-55.6	-70.9	-55.6	-76.9	-61.1
CPGR %		-16.86	-10.91	-19.45	-12.64	-18.58	-12.64	-21.72	-14.56

** Statistically significant (P value <0.001); ** Statistically not significant. Cellulase activity, expressed as µ moles of glucose /mg cellulose/h. The remaining notations are the same as in Table 1.*

Mean Cellulase Activity and Cellulose Digestion

Cellulase is a carbohydrase that digests the plant sugar cellulose into glucose monomers by breaking ß (1-4) linkages (Voet *et al*., 1988; Watanabe and Tokuda, 2010). The digestive system of silkworm substantially evolved a microbial-assisted cellulose synthesizing mechanism in the gut wall and cellulose hydrolyzing mechanism in the gut lumen. Being a structural polymer of mulberry leaf, the cellulose is made available to the silkworm through the mulberry diet and in the gut lumen it is digested by the endosymbiotic bacteria such as *Enterobacteriaceae; Protius vulgaris, Klebsiella pneumonia* and *Citrobacter freundii* (Kandylis *et al*., 2009; Pytelkova *et al*., 2009: Anand *et al*., 2010).

While, the cellulose levels recorded significant elevations throughout the fifth instar, the mean cellulase activity declined in the gut wall but remained constant in the gut lumen (Tables 7, 8 & Fig. 4). In ZDC, the gut wall cellulase recorded a mean activity level of 4.47 µ moles of glucose /mg cellulose / h and a negative CPGR of 16.86% during the fifth instar regime. But under the impact of nutrient diets, its activity further dropped to 3.69 µ moles (CPGR: -19.45%) in HFE, 3.79 µ moles (CPGR: -18.58%) in LFE and 3.65 µ moles (CPGR: -21.72%) in HLFE. But surprisingly, in the gut lumen the mean sucrase activity remained stable at 0.11 µ moles (CPGR: -10.91%) in control and experimental groups and that the nutrient diets have not caused any discernable change in its activity (Tables 7, 8 & Fig.4).

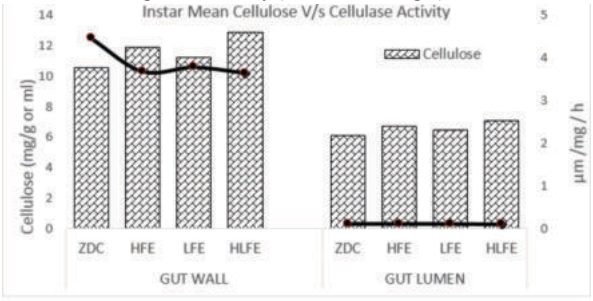

***Fig.4:** Synergistic effect of honey and lemon juice-enriched mulberry diets on cellulose levels and* cellulase activity *in the gut wall and gut lumen in B. mori during fifth instar larval development (Source: Tables 7 & 8)*

Nutrient Diets Reinforce Biomass Accumulation under Hutchinson's Investment Principle

The chief function of digestive metabolism is to supply nutrients and energy reserves for larval growth and metamorphosis in accordance with the Hutchinson's investment principle (HIP). As illustrated in chapter 3 on Silkworm Growth, the larval body mass recorded an increase of 3357% in ZDC, ~3986% in HFE, ~3914% in LFE and ~4071% in HLFE during fifth instar, probably in tune with its voracious feeding habit coupled with high power of digestibility (Hou *et al.,* 2010). Apparently, the biomass accumulation in silkworm depends on four factors; the quality of mulberry leaf, larval feeding behaviour, growth of gut vis-à-vis its digestive function. It has been demonstrated that the gut grows hypo-allometrically during this instar and achieves physiological plasticity in its size as the silkworm does not eat during pupation and even lack functional mouthparts during the adult phase (Blossmam-Myer and Burggren, 2010; Yeoh *et al.,* 2012; Grunert *et al.,* 2015). Needless to say the accumulation of digestive mass depends on the food supply, its digestion and absorption. As predicted by the HIP (Hutchinson *et al.,* (1997) and ratified by Siva Prasad (2022), the longer duration (7 days) of fifth instar not only allows the greater feeding time for the larva, but also facilitates increased biomass accumulation in body tissues for its utilization during metamorphosis. The current study analyzes the extent of mulberry mass conversion into the mass of proteins, carbohydrates, sucrose and cellulose in gut wall tissues during the fifth and final larval instar.

Protein Mass: The gut wall protein mass consists of over 96 proteins derived from the digestive metabolism. Apparently, it includes 22 proteins involved in digestion (eg. amylase, cellulase, hemicellulase, cellobiase, urease, invertase, trehalase, lipase, sucrase, xylanase, pectinase, trypsin and dipeptidase), 44 proteins involved in metabolism and energy production and 11 proteins involved in cellular transport, cell signaling, cytoskeleton formation, cell growth, immunity, heat shock treatment, muscle contraction, carcinoma control, carotenoid binding and antimicrobial activity (Kajiwara *et al*., 2005; Xia *et al*., 2007; Hui-Peng Yao *et al*., 2009; Zhang *et al*., 2011). Needless to say it has pre-determined functional roles in silk production and metamorphosis (Sarangi and Anitha, 2007). Quantitatively, the protein mass registered an instar mean mass of ~8 mg/g wet weight of gut wall tissue in ZDC under normal dietary conditions and showed increased turnover under nutritionally-enriched dietary conditions. Consequently, the protein mass was boosted to ~14 mg/g in HFE, ~12 mg/g in LFE and ~18 mg/g in HLFE (Table 1). The OGR, which represents the whole instar (V instar) growth trend and the CPGR, which represents day-wise growth trends within an instar also showed promising results. The ZDC recorded an OGR of ~312% and a CPGR of 26.59% during fifth instar and both these two rates were further enhanced in the other three nutrient groups. In HFE, the OGR was elevated by 220 percentage points (532-312%) and the CPGR by 9.39 percentage points (35.98-26.59%). In LFE, the OGR of protein mass was elevated by 131 percentage points (443-312%) and the CPGR by 5.99 percentage points (32.58 – 26.59%). The synergistic effect of honey and lemon juice was more pronounced on these growth rates. Evidently, the HLFE recorded a greater elevation of 383 percentage points (695-312%) in OGR and 7.84 percentage points (34.43-26.59%) in CPGR. (Table 1 & Fig. 5).

Carbohydrate Mass: The gut wall carbohydrate mass is formed by two storage sugars; trehalose and glycogen that are largely derived from the carbohydrate-rich mulberry diet. The dietary carbohydrate

mass, so obtained is mobilized as energy reserves for silk production and the maintenance of cocoon quality traits through the enhanced activity of α-amylase and other carbohydrases (Thompson, 2003; Shivakumar and Shamitha, 2011; Anandkumar and Sandhya, 2012; Kalaivani *et al*., 2013). Compared to protein mass, the digestive carbohydrate mass showed lower values in terms of both quantity and rate of accumulation. Quantitatively, it registered a mean value of ~10 mg glucose/g wet weight of gut wall tissue under normal dietary conditions, with an OGR of ~147% and a CPGR of 16.30% during fifth instar. Upon feeding silkworms with honey and lemon juice-enriched mulberry diets, the mean carbohydrate values were significantly enhanced to ~11 mg/g each in HFE and LFE and ~12 mg/g in HLFE (Table 3). Both OGRs and CPGRs were also elevated by the nutrient diets. In HFE, the OGR was elevated by 30 percentage points (177-147%) and the CPGR by 2.18 percentage points (18.48-16.30%). In LFE, the OGR of carbohydrate mass was elevated by 15 percentage points (162-147%) and the CPGR by 1.10 percentage points (17.40-16.30). The synergistic effect of honey and lemon juice was more significant on the growth of the protein mass. Evidently, the HLFE recorded a greater elevation of 43 percentage points (190-147%) in OGR and 3.10 percentage points (19.40-16.30%) in CPGR. Because of low OGRs and CPGRs, the carbohydrate mass accumulated in moderate quantities in the gut wall cells, compared to that of proteins (Table 3 & Fig. 5).

Sucrose Mass: Sucrose is a non-reducing disaccharide and the product of photosynthesis (Sumida and Ueda, 2007). Fresh mulberry leaf is the rich source of sucrose (~72 mg/g) that stimulates feeding behaviour in silkworm (Kuribayashi *et al*., 1990; Sumida and Ueda, 2007). Further, it is the main source of energy for metabolism in insects and its excess concentration is stored in the form of glycogen and trehalose in their body tissues (Thompson *et al*., 2002; Sumida and Ueda, 2007). Importantly, it functions as a cryoprotectant and membrane stabilizer and regulates the water flux across the gut wall in insects (Thompson, 2003). In the current study, the sucrose mass showed lower values compared to that of total proteins and carbohydrates. Quantitatively, it registered a mean mass of ~8 mg glucose/g wet weight of gut wall tissue in ZDC at an OGR of ~44% and a CPGR of 6.27% during fifth instar. Upon feeding silkworms with honey and lemon juice-enriched mulberry diets, the mean sucrose levels were reached ~10 mg/g in HFE, ~9 mg/g in LFE and ~11 mg/g in HLFE through higher growth rates (Table 5). The sucrose mass showed an increase of 50 percentage points (94-44%) in OGR and 5.39 percentage points (11.66-6.27%) in CPGR in HFE. In LFE, its OGR was elevated by 22 percentage points (66-44%) and the CPGR by 2.49 percentage points (8.76-6.27%). The synergistic effect of honey and lemon juice was more significant on the growth of the sucrose mass. Evidently, the HLFE recorded a greater elevation of 70 percentage points (114-44%) in OGR and 7.23 percentage points (13.50-6.27%) in CPGR (Table 5 & Fig. 5).

Cellulose Mass: Cellulose is the chief constituent of mulberry leaf and major dietary source of carbohydrates for the silkworm (Kandylis *et al*., 2009). Though, the cellulose is not an integral part of structure and function, the digestive system of silkworm evolved microbial-assisted cellulose synthesizing mechanism in the gut wall and cellulose hydrolyzing mechanism in the gut lumen. The gut of silkworm harbors a community of cellulose synthesizing microbes like *Escherichia coli* and *Pseudomonas, which* do not utilize the dietary cellulose, but synthesize their own cellulose de novo within the gut wall cells that protects the sensitive gut wall cells from the cellular damage that might occur due to osmotic imbalances caused by the continuous presence of large quantities of undigested dietary cellulose in the gut lumen. (Romling, 2002; Anand *et al*., 2010). Obviously, the accumulation of cellulose in gut wall cells helps in establishing a cellulose gradient in midgut that helps in withstanding osmotic pressure differences between the extra cellular (gut lumen) and intracellular (gut cells) spaces apart from sustaining the load bearing function of gut. Thus, the silkworm digestive system acts as a dual functional system for the synthesis and hydrolysis of the cellulose that are synergistically modulated by the host organism and the symbiotic microbes (Hongoh *et al*., 2008; Liu *et al*., 2008). The microbial-derived cellulose thus accumulates in small quantities in the gut wall cells. In the present case, the silkworm gut wall recorded a negligible quantity of cellulose mass, equivalent to ~11 mg glucose / wet weight of gut wall tissue in ZDC under normal dietary condition, with an OGR of ~23% and a CPGR of 3.44% during fifth instar. Upon feeding silkworms with honey and lemon juice-enriched mulberry diets, the microbial-assisted cellulose synthetic machinery has been geared-up, facilitating more cellulose synthesis. Consequently, the mean cellulose levels were elevated to ~12 mg/g each in HFE, ~11 mg/g in LFE and ~13 mg/g in HLFE and registered higher growth rates (Table 7). Accordingly, in HFE, the OGR was elevated by 25 percentage points (48-23%) and the CPGR by 3.34 percentage points (6.78-3.44%). In LFE, its OGR was elevated by 10 percentage points (33-23%) and the CPGR by 1.46 percentage points (4.90-3.44%). The synergistic effect of honey and lemon juice was more significant on microbial cellulose synthesis. Evidently, the HLFE recorded a greater elevation of 41 percentage points (64-23%) in OGR and 5.15 percentage points (8.59-3.44%) in CPGR. Evidently, the gut wall maintains of low cellulose levels, when compared to other assimilated digestive products (Table 7 & Fig. 5).

The current study demonstrates that the silkworm accumulates more protein mass in its gut wall, compared to that of total carbohydrates, sucrose and cellulose and this is in consonance with our earlier findings (Madhavi and Siva Prasad, 2022). Further, this occurs in a circadian fashion and is fine-tuned by the 12 h light and 12 h dark free running time (Bhuvaneswari and Siva Prasad, 2012a, 2012b; Bhuvaneswari *et al.,* 2013). The findings of the present investigation also reflect that the digestive mass accumulation in the gut wall tissue follows the timelines set by the critical larval body size determinants as evidenced by higher growth rates on day-3 or day-5 of fifth instar in respect of all the four digestive masses examined. Necessarily, the rate of biomass accumulation is probably interrupted by some developmental cues such as the timing of attainment of critical and threshold sizes by the fifth instar silkworm that might contribute to the incapacity of the gut to transport nutrients that imposes additional restrictions on larval growth (Grunert *et al.,* 2015).

CONCLUSIONS

The digestive system is the route through which the mulberry diet is ingested, digested, absorbed and assimilated in the larval body tissues. The present investigation indicates four important features of digestive metabolism in *B. mori*. First, the mulberry diet is digested both in the midgut wall and midgut lumen compartments and its digestibility is more pronounced in the former and less intensive in the latter. Second, the protein digestion proceeds on an elevatory note, while carbohydrates are digested at a low steady state throughout the fifth instar. Third, the honey and lemon juice-enriched mulberry diets boosted the digestibility of protein foods, but did not affect the digestibility of carbohydrate foods. Fourth, the digestive biomass accumulates in the gut wall tissue in accordance with the Hutchinson's investment principle and it is fine-tuned by the timing of the acquisition of critical body size determinants. As suggested by Ueda (1982), the digestive anabolism (synthetic accumulation of metabolites in gut wall cells) increases, while the digestive catabolism (digestibility of nutrients in gut lumen) decreases during fifth instar development.

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